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Metabolic characterization of grapevine leaves: first clues towards biomarkers discovery

Mestrado em Bioquímica
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Declaração

De acordo com o disposto no artigo n.º 19 do Regulamento de Estudos de Pós – Graduação da Universidade de Lisboa, Despacho n.º 2950/2015, publicado no Diário da República, 2.ª série — N.º 57 — 23 de março de 2015, foram incluídos nesta dissertação os resultados do artigo:

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Em cumprimento com o disposto no referido despacho, esclarece-se ser da minha responsabilidade a execução das experiências que estiverem na base dos resultados apresentados (exceto quando referido em contrário), assim como a interpretação e discussão dos mesmos.

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Table of contents

Resumo

A videira (*Vitis vinifera* L.) é atualmente a árvore de fruto mais cultivada em todo o mundo devido à sua importância económica na indústria vinícola. Em 2014, a área mundial coberta por vinhas para produção de uvas, passas de uva, vinho e outros produtos foi de 7375 kha (Relatório anual da Organização internacional de vinha e vinho). A União Europeia é líder mundial na produção de vinho, tendo quase metade da área vinícola total (Relatório Anual e Estatísticas da produção vinícola - Rede Global de Informação Agrícola, 2014). Em relação à produção de vinho, França, Itália, Espanha e Argentina são atualmente os cinco países com maior produção. Por outro lado, a China, Turquia, Irão e Índia são os maiores produtores mundiais de uvas frescas para consumo. Portugal é o décimo produtor de vinho a nível mundial e o quinto na Europa, tendo este sector uma elevada importância estratégica para a economia do País, representando cerca de 890 milhões € em exportações só em 2013 (dados de Global Agricultural Information Network, 2013). De todos os produtos vitícolas, e apesar das uvas e do vinho serem maioritariamente os mais comercializados, alguns países como a Turquia, Arábia Saudita, Grécia, Bulgária, Roménia e Vietnam estão a cultivar algumas espécies de videira especificamente para a produção de folhas para consumo na alimentação. Em Portugal, as folhas de algumas cultivares de videira começaram já a ser analisadas para a inclusão na dieta, devido ao seu conteúdo antioxidante e compostos fenólicos. Os seus benefícios para a saúde têm sido cada vez mais estudados, tendo-se demonstrado que este produto pode ser utilizado, por exemplo, para o tratamento de dores crónicas, processos inflamatórios e pressão arterial elevada. Estas propriedades, o valor nutricional, o sabor e a qualidade são devidos a uma diversidade de compostos secundários como compostos fenólicos, ácidos orgânicos, lípidos e glúcidos.

As cultivares de *Vitis vinifera* normalmente utilizadas na produção de vinho são suscetíveis ao míldio, uma doença extremamente destrutiva causada pelo fungo *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni. Esta doença foi acidentalmente introduzida na Europa durante o século XIX e continua a ser uma das mais destrutivas doenças que afetam as vinhas neste continente. Estudos realizados indicam que, em interações compatíveis, este fungo poderá controlar o metabolismo das células hospedeiras, permitindo assim a sua sobrevivência e proliferação, promovendo a destruição completa das vinhas e consequentemente significantes perdas económicas. Em 2014, a colheita de uva em Portugal diminuiu cerca de 5.7%, devido a más condições meteorológicas na região Norte e devido ao aparecimento de míldio nas regiões do Centro e Sul, que subsistiu apesar dos tratamentos com fungicidas

aplicados. Algumas estratégias têm sido implementadas de forma a evitar a ameaça causada pelo *P. viticola*, incluindo o uso intensivo de fungicidas e fitoquímicos. Estas abordagens não são eficazes, nem compatíveis com um desenvolvimento sustentável, nem seguras para a saúde pública. Para além disto, as folhas podem ser incluídas na alimentação e a sua exposição a estes produtos químicos torna a sua descontaminação e certificação para a comercialização ainda mais difícil.

Algumas espécies Americanas e Asiáticas de *Vitis* são resistentes ao míldio, pelo que uma possível alternativa na prevenção da infeção pelo míldio é a criação de alguns híbridos intra-específicos. Esta última abordagem é claramente mais eficaz e sustentável, particularmente se for acoplada à seleção de características com interesse económico de cultivares de videira locais, permitindo a preservação das propriedades desejadas e únicas do vinho local. No entanto, um programa bem-sucedido de reprodução de plantas melhoradas com características de resistência ao míldio requer, não só uma compreensão dos mecanismos de resistência inata de cultivares contra os fungos, mas também a identificação de biomarcadores de resistência. De entre estes, os biomarcadores metabólicos podem revelar-se particularmente úteis. Inúmeros trabalhos científicos nas áreas de transcritómica, proteómica e metabolómica foram publicados na última década com abordagens para a caracterização de resistência ao míldio. Uma análise metabólica dos diversos produtos da videira é de elevada importância visto que as plantas contêm um metaboloma único que varia com as condições ambientais, o desenvolvimento da planta e infeções de patogénicas. Em relação às folhas de videira, visto que é o primeiro órgão que o *P. vitícola* infecta, estas podem possuir na sua composição biomarcadores de resistência ou suscetibilidade contra este patogénio.

Normalmente, um estudo metabólico é realizado recorrendo a técnicas como a Ressonância Magnética Nuclear (RMN) e Espectrometria de Massa (MS) ou à combinação destas técnicas a processos de separação cromatográficos como a Cromatografia Gasosa (CG) ou Líquida (LC). Contudo, os limites de deteção por RMN são baixos e apesar da combinação de MS com CG e LC possibilitar a deteção de um elevado número de compostos, os processos cromatográficos demoram muito tempo e normalmente necessitam de vários passos de limpeza do equipamento. Uma solução será a utilização de *Fourier Transform Ion Cyclotron Resonance* acoplado a um espectrómetro de massa (FT-ICR-MS) visto que é um equipamento que não só combina a elevada resolução com a elevada exatidão de massa como também permite uma rápida e fácil aquisição de resultados.

Esta dissertação teve como foco principal a caracterização metabólica de folhas de *Vitis vinifera* cultivar Pinor noir. O primeiro objetivo foi estabelecer um protocolo de extração de metabolitos compatível com uma análise de espectrometria de massa com elevada resolução (Capítulo II). Para isso, foi desenvolvido um protocolo de extração de metabolitos com uma mistura de solventes dando origem a quatro frações (metanólica, aquosa, orgânica e acetonitrilo) que foram analisadas por FT-ICR-MS. A análise destas quatro frações foi efectuada em modo positivo e negativo, permitindo identificar no total, respetivamente, 634 e 133 massas únicas.

Após o estabelecimento de um protocolo de extração otimizado, o segundo objetivo foi a análise do metaboloma das folhas de videira da cultivar Pinot noir e a importância destes compostos na saúde humana (Capítulo III). Os compostos identificados foram anotados e divididos em oito classes metabólicas principais (lípidos, glúcidos, ácidos nucleicos, compostos fitoquímicos, compostos heterocíclicos, ácidos orgânicos e derivados, benzenóides e outros) e estas posteriormente em classes secundárias e terciárias quando necessário. Do metaboloma anotado, a classe mais representada neste estudo foi a classe dos lípidos com 67%, seguida dos compostos fenólicos (13%), ácidos orgânicos (7%) e glúcidos (3%). É importante salientar que na classe dos compostos fenólicos, foi detetada pela primeira vez em folhas da cultivar Pinot noir uma antocianina acetilada. Para além disso, foi ainda realizada uma análise das vias metabólicas utilizando uma ferramenta *online* pública, *Kyoto Encyclopedia of Genes and Genomes* (KEGG), de modo a perceber a cobertura dos nossos resultados e identificar compostos importantes.

Finalmente, o terceiro objetivo foi comparar a nível metabólico dez espécies e cultivares, resistentes e suscetíveis ao míldio, identificar compostos presentes apenas num grupo de videiras resistentes ou suscetíveis, permitindo desta forma diferenciar os dois grupos e integrar essa informação metabólica com a quantificação de transcritos, de forma a definir marcadores de resistência (Capítulo IV). No total foram identificados nove compostos presentes apenas no grupo de videiras resistentes ou suscetíveis ao míldio. Foram também incluídos na análise sete compostos descritos na literatura como possíveis marcadores de resistência ou com padrões de acumulação em diferentes videiras. A quantificação da expressão de genes que codificam para enzimas de síntese ou degradação para estes compostos foram analisados por reação de polimerase de cadeia em tempo real (qPCR). Para este trabalho a nossa referência e o principal modelo de estudo foi a cultivar Pinot noir, sendo a expressão dos genes desta cultivar considerada como controlo para todos os outros. Para além disto, como a expressão de genes de interesse tem de ser normalizada com genes de

referência, a estabilidade de onze genes foi avaliada e os três genes mais estáveis foram considerados utilizados para normalizar os dados.

Cada capítulo desta tese foi escrito como um artigo científico e cada possui o seu próprio resumo, introdução, materiais e métodos, resultados e discussão, conclusão, agradecimentos e referências bibliográficas.

Esta abordagem de biologia de sistemas é um ponto de partida para elucidar quais as bases moleculares inerentes à resistência e suscetibilidade das videiras e, quem sabe, a longo prazo, utilizar estes metabolitos associados à resistência no desenvolvimento de ensaios de biomarcadores para utilização em futuros programas de melhoramento e análise de linhagens.

Palavras-chave: *Vitis vinifera*; *Plasmopora viticola*; downy midew; Metabolómica; FT-ICR-MS; qPCR; expressão de genes.

Summary

Grapevine (*Vitis vinifera* L.) is the most important fruit crop in the world due to its numerous food products, namely fresh and dried table grapes, wine grapes, leaves and intermediate products. Traditional premium cultivars of wine and table grapes are highly susceptible to various diseases, particularly to downy mildew. Grapevine downy mildew is caused by the biotrophic oomycete *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni and is one of the most destructive diseases in *Vitis vinifera*. In Europe, disease management became one of the main tasks for viticulture, being the current strategy, for downy mildew disease control, the massive use of fungicides and pesticides in each growing season. This practice has several associated problems, from the environmental impact to the economical level, and even in human health. The alternative approach is the introduction of resistant grapevine varieties, clearly most effective and sustainable, particularly if coupled to the selection of desirable traits from local grapevine cultivars. However, a successful breeding program of grape plants with increased resistance traits against downy mildew requires, not only an understanding of the innate resistance mechanisms of cultivars against fungi, but also the identification of biomarkers of resistance (or susceptibility). Among these, metabolic biomarkers may prove particularly useful, not only because they can be determined in a high throughput way but, above all, because metabolites provide accurate metabolic state definition.

The first part of this thesis (Chapter II) focuses on the establishment of an appropriate metabolite extraction protocol for *Vitis vinifera* cultivar Pinot noir grapevine leaves, compatible with high resolution mass spectrometry. Different extraction protocols with different solvent mixtures were tested and the metabolic analysis was performed using direct infusion Fourier-transform ion cyclotron-resonance mass spectrometry (DI-FTICR-MS). A mixture of methanol/water/chloroform was the best extraction mixture, allowing a higher metabolite extraction yield, reproducibility and metabolome cover. Four fractions were obtained (methanol, water, organic and acetonitrile), which were analysed by FT-ICR-MS, using electrospray ionization (ESI) in both ionization modes (positive [ESI^+] and negative [ESI^-]). In total we identified 634 and 144 unique masses by, respectively, ESI^+ and ESI^-. The metabolic coverage of the fractions was compared for ESI^+ and ESI^- and only 59 masses were common in both ionization modes. This extraction protocol allowed the extraction of polar and non-polar compounds, covering all major classes found in plants and increasing metabolome coverage.

After the establishment of an optimized and efficient extraction protocol, metabolite annotation of grapevine leaves from cultivar Pinot noir was performed, not only with the

purpose of obtaining the most comprehensive metabolite analysis in *Vitis vinifera*, but also for metabolite characterization associated with leaves' high nutritional value, taste and health benefits (Chapter III). Indeed, grapevine leaves are considered a healthy food due to their human health benefits, mainly for their content in a wide range of phenolic compounds. The metabolome of *Vitis vinifera* cultivar Pinot noir grapevine leaves was annotated and the identified compounds were classified into eight major metabolic classes (lipids, carbohydrates and conjugates, nucleic acids, phytochemical compounds, heterocyclic compounds, organic acids and derivatives, benzenoids and others), secondary and if needed into third metabolic classes. Lipids were the most represented class with 67% of the metabolome identified followed by phytochemical compounds (13%), organic acids (7%) and carbohydrates (3%). Each of these classes was further associated to human health benefits, highlighting the benefits of grapevine leaves consumption. Noteworthy, thirteen mostly used pesticides were also found in grapevine leaves, being therefore essential the development of new methodologies for quality assessment and safe commercialisation.

The comprehensive metabolite analysis from *Vitis vinifera* leaves was the starting point for the identification of biomarkers of resistance or susceptibility against the downy mildew. In order to identify compounds present only in susceptible or in resistant grapevines, ten *Vitis vinifera* species and cultivars, were compared at the metabolite level (Chapter IV). The metabolite extraction protocol previously optimized was used, but only the methanol fraction (which contained some of the most relevant compounds involved in defence mechanisms) was analysed by DI-FTICR-MS using ESI^+ and ESI^-. A first analysis revealed nine masses distinctly identified (6 in ESI^- and 3 in ESI^+) that might be resistant- or susceptible-specific. The goal was to integrate this information with transcript quantification. Hence, two of these compounds were selected, together with seven other reported in literature as involved in defence mechanisms and their metabolic pathways were identified. The expression profile of several biosynthesis and/or degradation enzymes coding genes was performed by Real-time Polymerase Chain Reaction (qPCR). To have success in this analysis, qPCR studies require as internal controls one or more reference genes, for all the samples analysed. Hence, eleven possible reference genes were tested and normalization of the qPCR results from the enzyme coding genes was made using the three most stable reference genes. This systems biology approach, based on the integration of the metabolic profiling and transcriptomics will enlighten the molecular basis of the differences between susceptible and resistant cultivars.

Each chapter was written as a scientific article and has its own abstract, introduction, materials and methods, results and discussion, conclusion, acknowledgements and references.

This thesis is a starting point for, in the long run, use these resistance-associated metabolites in the development of streamlined biomarker assays to assist future breeding programs and introgression line analysis.

Keywords: Vitis vinifera; Plasmopora viticola; downy mildew; Metabolomics; FT-ICR-MS; qPCR; gene expression.

Abbreviations

1D NMR	One dimension Nuclear Magnetic Resonance
60S	60S ribosomal protein L18
ACT	Actin
ANR	Anthocyanidin reductase
AP2M	Adaptor protein-2 MU-adaptin
C3H	*p*-coumarate 3-hydroxylase
COMT	Caffeic acid *O*-methyltransferase
DI-FTICR-MS	Direct Infusion Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
EF1α	Elongation factor 1-alpha
ESI^-	Electrospray Ionisation in Negative mode
ESI^+	Electrospray Ionisation in Positive mode
F3'5'H	Flavonoid 3',5'-hydroxylase
FatB	Fatty acyl-ACP thioesterase B
Fd-GOGAT	Ferredoxin-dependent Glutamate synthase
FUM1	Fumarate hydratase I
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gas Chromatography
IMPL1	Myo-inositol monophosphatase
LAR2	Leucoanthocyanidin reductase 2
LC	Liquid Chromatography
m/z	mass to charge ratio (Unit: Thomson)
MS	Mass Spectrometry
NADH-GOGAT	NADH-dependent Glutamate synthase
NMR	Nuclear Magnetic Resonance
PCR	Polymerase Chain Reaction
PsaB	Photosystem I P700 chlorophyll a apoprotein A2

qPCR	Real time Polymerase Chain Reaction (quantitative PCR)
SAND	SAND family protein
SPE	Solid Phase Extraction
TTC7B	Tetratricopeptide repeat protein 7B
UBQ	Ubiquitin-conjugating enzyme
UFGT	UDP-glucose:flavonoid 3-*O*-glucosyltransferase
α-*TUB*	Alpha-tubulin 3-chain
β-*TUB*	Beta-tubulin 1-chain

Units

Symbol	Name	Equivalent Units
°C	Celsius	
Ω	ohm	
µg	microgram	10^{-6} g
µL	microliter	10^{-6} L
Da	Dalton	
g	Centrifuge force	
g	gram	
h	hour	
Kha	Thousands of hectares	
L	Litre	
m/z	mass-to-charge ratio	
min	minute	60s
mL	millilitre	10^{-3} L
ms	millisecond	
ppm	parts per million	1/1000000
S	second	
V	volt	Ω^{-1}
v/v/v	Volume/Volume/Volume	
W	Water fraction	
ρg	picogram	10^{-12} g

Chapter I

Introduction

1. The grapevine

Vitis vinifera L. (grapevine) is a member of the Vitaceae family which englobes about 60 wild *Vitis* species globally distributed (Feechan et al., 2011; Terral et al., 2010). The cultivated *Vitis vinifera* comprises up to 5000 cultivars used for wine production and grapes commercialization (Ali et al., 2010). Based on both archaeological and historical studies, it is clear that the cultivation and domestication of grapevines, the production of wine and commercialization of grapevine products has always been an important part of the development of human culture (Ali et al., 2010; Terral et al., 2010). Nowadays, *Vitis vinifera* L. is one of the most important and cultivated fruit plant in the world due to its food products, with a major economic, environmental and medical importance. As a result of its easy cultivation and numerous applications, in 2014, the global vine area for fresh and dried table grapes, wine grapes and intermediate products was 7375 kha (Fig. I. 1) (International Organisation of Vine and Wine (OIV), 2015).

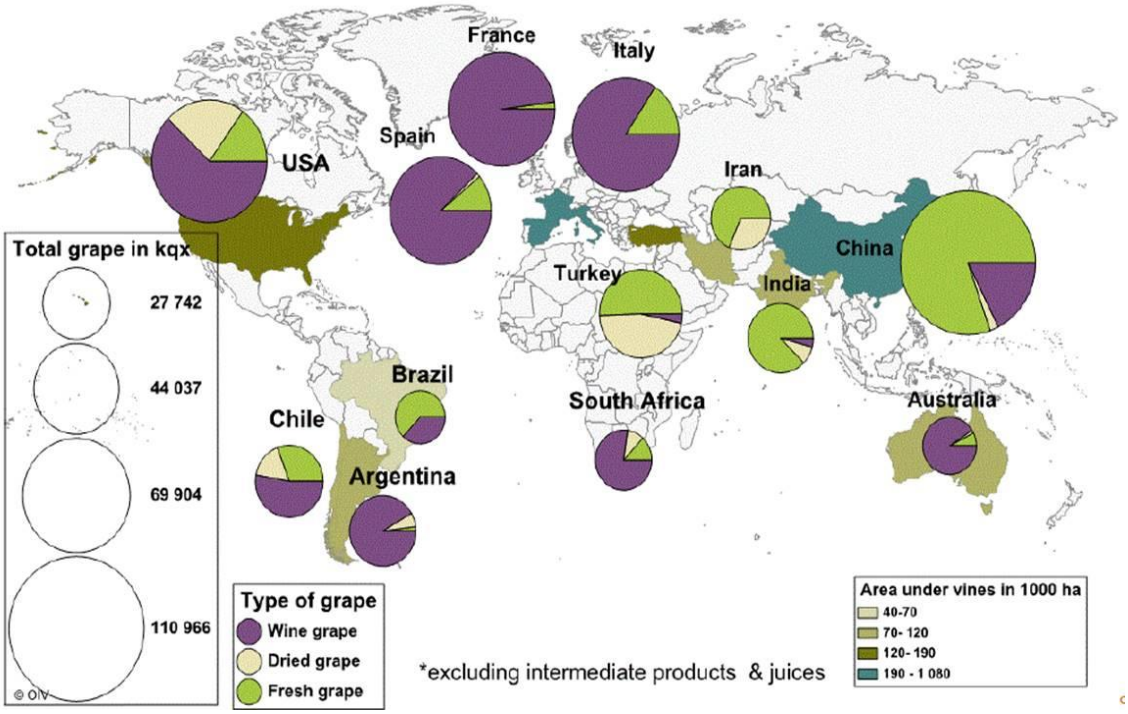

Fig. I. 1 - Major grape producers worldwide. Image from 2015 World vitiviniculture situation report (International Organisation of Vine and Wine (OIV), 2015).

Concerning wine production, France, Italy, Spain, USA and Argentina are currently the top 5 grape wine production countries. On the other hand, China, Turkey, Iran and India represent the major producers of fresh grapes worldwide. In 2014, 55% of the total share of grapes was used for wine, 35% and 8% were used, respectively, for fresh and dried

consumption, leaving 2% for other intermediate products and juices (International Organisation of Vine and Wine (OIV), 2015). In Portugal, the wine industry is highly strategic for the country's economy, accounting for 890 million euros per year of exports (Bettini, 2014). Portugal is the tenth world wine producer, and the fifth in Europe, with 14 wine producing regions: Península de Setúbal, Lisboa, Tejo, Bairrada, Vinho Verde, Dão, Távora-Varosa, Porto-Douro, Trás-os-Montes, Beira interior, Alentejo, Algarve, Açores and Madeira (Fig. I. 2), corresponding to around 224 kha surface under vineyards in 2014 (International Organisation of Vine and Wine (OIV), 2015; http://www.winesofportugal.info/)

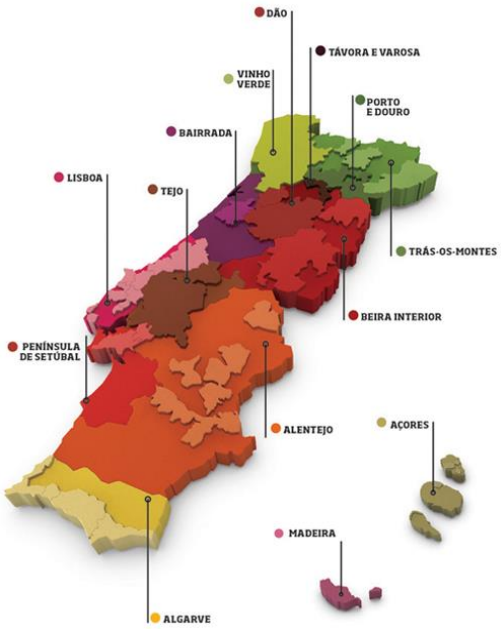

Fig. I. 2 - Representation of the Portuguese wine producing regions (image from http://www.winesofportugal.info/, used with permission for non-commercial uses).

Although wine and grapes are the major commercialized grapevine products, in some countries such as Turkey, Saudi Arabia, Greece, Bulgaria, Romania and Vietnam some grapevine varieties are especially grown for fresh and brined leaves consumption (Koşar et al., 2007; Rizzuti et al., 2013; Sat et al., 2002). This change in the use of vines led some top wine producer countries, like Italy, to gain interest into this grapevine product (FAO Statistical Yearbook, 2014; Rizzuti et al., 2013). In Portugal, grapevine leaves from Portuguese cultivars started to gain interest for their inclusion in the human diet, due to their content in antioxidants and phenolic compounds (Lima et al., 2016). The health benefits of grapevine leaves have been extensively demonstrated in inflammatory disorders, pain, bleeding and high blood pressure treatments. Also, their anti-oxidant properties have been described to protect and retard oxidative processes (Ali et al., 2010; Dani et al., 2010; Ledesma-Escobar and Luque

de Castro, 2015; Orhan et al., 2007; Pari and Suresh, 2008; Fleming, 2000). These properties, nutritional value, taste and quality are due to a diversity of secondary bioactive metabolites like phenolic compounds, organic acids, lipids and carbohydrates and therefore, grapevine leaves are considered a healthy food (Lima et al., 2016).

A major risk to *Vitis vinifera* cultivars, commonly used for grape, wine or leaf production is their susceptibility to many diseases. Nowadays, the most threatening grapevine pathogens are those causing powdery [*Uncinula necator* (Schw.) Burr] and downy mildew [*Plasmopara viticola* (Berk. et Curt.) Berl.et de Toni, Fig. I. 3] (Ali et al., 2009; Figueiredo et al., 2008; Gessler et al., 2011; Polesani et al., 2010).

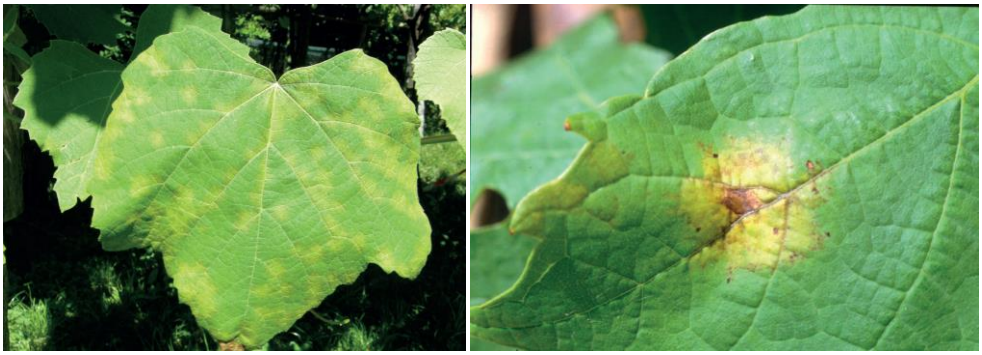

Fig. I. 3 - Grapevine leaf surfaces infected with Plasmopara viticola, the causing agent of downy mildew [image from (Gessler et al., 2011), (CC BY-NC-ND 2.5)].

2. The downy mildew

Grapevine downy mildew was accidentally introduced in Europe during the XIX century and is still one of the most destructive grapevine diseases in this continent (Figueiredo et al., 2008). The pathogen affects the leaves, shoots and fruits, reducing berry quality and yield, with significant production losses (up to 75 to 100% of an entire crop). For example, in 2011 in Portugal, wine production losses due to downy mildew disease increased around 50% in Douro, 30% in Alentejo and 40% in Ribatejo and Península de Setúbal, comparing with the year before. In Minho, which is the most commonly affected region, the loss was only 20% due to a better control and supervision of vineyards (Falcão, 2011). Currently, the strategy used is the indispensable application of powerful fungicides and pesticides. These approaches are not cost-efficient, environmental friendly and safe for the human health since these products contaminate the soils, drinking water and food (Cabras and Angioni, 2000; Lamichhane et al., 2015). Moreover, leaves have the disadvantage of greater exposure to them, when compared

with berries (Ali et al., 2009), being their decontamination for commercialisation still a barrier. The alternative strategy for disease control is the development of breeding programs between wild *Vitis* species (resistant to downy mildew) and *V. vinifera* (susceptible) towards the development of new cultivars with good berry quality and a high degree of resistance against downy mildew. These breeding approaches offer forward-looking perspectives for an environmental friendly and sustainable viticulture. Several American *Vitis* species, including *V. riparia* and *V. labrusca*, exhibit resistant traits with different levels of resistance to *P. viticola* (Gessler et al., 2011) and have been successfully used in breeding programs. For example, *V. vinifera* cultivar Regent, developed from European (*V. vinifera*) and American vine species, possesses a broad resistance to the most significant fungal diseases, including downy mildew (Gessler et al., 2011). However, despite all the advances, recent reports documented the emergence of *P. viticola* resistance-breaking isolates in Europe, highlighting the need to deepen our knowledge on this interaction and to search for alternatives (Peressotti et al., 2010). Moreover, a successful breeding program of grape plants with increased resistance traits against downy mildew are time-consuming and requires, not only an understanding of the innate resistance mechanisms of cultivars against fungi, but also the identification of resistance-related biomarkers. So, the discovery of metabolic biomarkers will allow a quick and accurate identification of the seedlings that inherited the resistant trait soon after germination and to understand the complex resistance mechanism of some *Vitis vinifera* cultivars.

3. Metabolomics in the search for biomarkers

Metabolomics is a widely used approach in systems biology, alongside other "omics" like genomics, transcriptomics or proteomics. The analysis of metabolites in grapevine products is of upmost importance mainly because plants contain a unique metabolome that change with the environment, the development and upon pathogen infections (Fernie et al., 2004). Previous studies have unravelled the possibility that the leaf surface may be the most reliable source of biomarkers for resistance or susceptibility against pathogens (Batovska et al., 2009, 2008). These molecular biomarkers of resistance may be used as tools for breeders to easily select resistant plants in nurseries and for viticulturists to monitor crops.

A metabolic profiling study usually includes Nuclear Magnetic Resonance (NMR) or Mass Spectrometry (MS). NMR provides structural information, good reproducibility and quantitative aspects, but the limits of detection remain very low and some lipids can overlap

and cover interesting metabolite peaks (Becker et al., 2013; Wu et al., 2008). On the contrary, MS has facilitated simultaneous detection and quantification of a much larger number of metabolites and provides structural information through fragmentation experiments in the absence of commercially available standards (Becker et al., 2013). However, there are two main problems with high-throughput MS, especially with plants. First, is the obtained data which are a series of peaks without identification limiting the capability for metabolite identification (Iijima et al., 2008); second, some authors believe that a single analytical technique does not provide sufficient information on the metabolome of the sample under investigation (Rizzuti et al., 2013). Therefore, a combination of different techniques may be required to improve this coverage. For this matter, analysis of plant metabolites is still often performed using either specific enzyme assays or chromatographic separations such as Gas Chromatography (GC) or Liquid Chromatography (LC) usually coupled with MS (Lindon and Nicholson, 2008; Roessner et al., 2000). Although powerful, chromatographic techniques have some drawbacks. For example, the application of these analytical methods can only be applied to mixtures of low complexity with often clean-up steps (Roessner et al., 2000), the detection of non-volatile secondary metabolites is very difficult (Iijima et al., 2008) and in order to make various classes of compounds accessible for analysis, they undergo a complex series of extractions and chemical modification steps, which are time consuming (Roessner et al., 2000). Therefore, new approaches should be developed that combine high sensitivity and specificity with the potential to overcome all of the problems that have been described.

Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR-MS) is a technique not commonly used for plant metabolomics that presents a huge potential. FTICR is the fastest and the most powerful tool to fingerprint complex samples, especially for non-targeted metabolic analysis, due to a rapid screening and fast data acquisition rate (Aharoni et al., 2002). This MS system provides extremely high mass resolution (more than 1,000,000) and a mass accuracy often below 1 ppm (Brown et al., 2005; Fiehn, 2002; Han et al., 2008). Moreover, this approach allows the sample analysis by direct infusion, ensuring that complex metabolite samples are rapidly analysed in a high-throughput way, eliminating time-consuming separation steps (Kirwan et al., 2014).

So far, most of the plant metabolomics studies use GC-MS as a method of choice. This approach allowed the identification of 150 compounds in potato (*Solanum tuberosum*) tuber tissue (Roessner et al., 2000) and 326 metabolites in *Arabidopsis thaliana* leaves (Fiehn, 2002). In grapevine research, the main focus in metabolomics studies has been given to grape growth, development and ripening mainly due to the interest to understand the physiological

and biochemical events that determine grape and wine quality (Cuadros-Inostroza et al., 2016; Smart et al., 2006). Thus, most of such studies are limited to the metabolome analysis of grapevine berries of a determined cultivar (Deluc et al., 2007; Fortes et al., 2011; Grimplet et al., 2009; Zamboni et al., 2010), or to a particular kind of stress condition (Ali et al., 2012; Batovska et al., 2009, 2008; Hong et al., 2012). These were performed using NMR spectroscopy and based on the analysis of a single extract from leaves. In this case, albeit using 1D and 2D NMR techniques, the number of metabolites identified was no more than 30 (Ali et al., 2009). More recently, the application of GC-MS analysis to grapevine leaves allowed the detection of around 100 metabolites (Batovska et al., 2008). Using mass spectrometry coupled to liquid chromatography (LC-MS), although the identification and quantification of grapevine metabolites was possible, there were only identified 135 primary metabolites (sugars, amino acids, organic acids and amines) in a 30-minutes hydrophilic interaction LC run (Gika et al., 2012). FTICR started to be used in grapevine targeted metabolomics studies, mainly in negative ionization mode to ensure the detection of phenolic compounds (Daayf et al., 2012). However, the total of metabolites that can be identified and quantified using only one ionization mode falls well short of the total cellular metabolites. For a well coverage of the metabolome, a more thorough analysis using both positive and negative ionization modes is needed.

4. Aims

This master project's focus was the metabolic characterization of grapevine leaves.

The first aim was the establishment of an appropriate metabolite extraction protocol from grapevine leaves, compatible with high resolution mass spectrometry analysis. Hence, different extraction protocols with different solvent mixtures were tested and direct infusion (DI)-FTICR-MS was used.

Following the establishment and optimization of the extraction protocol, the second goal was to analyse at a metabolite level *Vitis vinifera* Pinot noir leaves, a highly important *Vitis vinifera* in wine making, using DI-FTICR-MS. Moreover, a metabolic pathway profiling using KEGG pathways tools was done to see the metabolic pathway coverage and to identify important compounds.

Finally, the third objective was to analyse a collection of metabolite datasets from several vine species and cultivars, resistant and susceptible to downy mildew, to understand which key pathways may be involved in grapevine innate resistance or susceptibility to this pathogen. This metabolomic information was integrated with transcript quantification in order to define metabolic markers of resistance that can be used as molecular screening traits in grapevine breeding programs.

5. References

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Chapter II

Metabolite extraction method for grapevine leaves

Maia, M., Monteiro, F., Sebastiana, M., Marques, A. P., Ferreira, A. E. N., Freire, A. P., Cordeiro, C., Figueiredo, A., Sousa Silva, M. (2016). Metabolite extraction for high-throughput FTICR-MS-based metabolomics of grapevine leaves. EuPA Open Proteomics, 12, 4-9.

1. Summary

In metabolomics there is an ever-growing need for faster and more comprehensive analysis methods to cope with the increase of biological studies. Direct infusion Fourier-transform ion cyclotron-resonance mass spectrometry (DI-FTICR-MS) is used in non-targeted metabolomics to obtain high-resolution snapshots of the metabolic state of a system. In any metabolic profiling study, the establishment of an effective metabolite extraction protocol is paramount. We developed an improved metabolite extraction method, compatible with DI-FTICR-MS-based metabolomics, using grapevine leaves. This extraction protocol allowed the extraction of polar and non-polar compounds, covering all major classes found in plants and increasing metabolome coverage.

2. Introduction

Grapevine (*Vitis vinifera* L.) is the most widely cultivated and economically important fruit crop in the world, mainly due to the wine industry. Many grapevine varieties are also grown for their use as food products, not only for table grapes, but also for the consumption of their leaves. Due to their astringent and hemostatic properties and phenolic composition, vine leaves are considered a healthy food and are consumed in several countries, including Saudi Arabia, Turkey and Greece (Koşar et al., 2007). The biochemical composition of both grapes and leaves is determinant for their nutritional value and taste. Furthermore, some authors believe that the most reliable source of biomarkers for resistance or susceptibility against pathogens is the leaf surface and the polar extracts from defatted leaf tissues (Batovska et al., 2009, 2008). Hence, the analysis of the compounds present in leaves is of utmost importance. This is particularly relevant when concerning plants, which are biochemically highly complex and contain a unique metabolome that change with the environment, the development and upon pathogen infections (Fernie et al., 2004).

So far, most of the metabolite studies in grapevine were performed by nuclear magnetic resonance (NMR) spectroscopy and were based on the analysis of a single extract from leaves (Ali et al., 2012; Figueiredo et al., 2008). In these studies by NMR, larger amounts of initial plant material are required (between 25 and 50 mg), the limit of detection is around 10 µM and even using 1D and 2D NMR techniques, and the number of metabolites identified is usually less than 20. More recently, mass spectrometry coupled to liquid chromatography (LC-MS) has been used in the identification and quantification of grapevine metabolites (Gika et

al., 2012). Although this methodology is more sensitive, only 135 primary metabolites (sugars, amino acids, organic acids and amines) were identified and quantified in a 30-minutes hydrophilic interaction LC run coupled to a triple quadrupole mass spectrometer (Gika et al., 2012).

To achieve higher sensitivity and maximum metabolome coverage, we resort to mass spectrometry using high-resolution and high-mass accuracy instruments, based on Fourier transform technology. The sensitivity of this methodology is much higher (typically ρg level) and different fractions can be analyzed (from aqueous to organic extractions) (Pan and Raftery, 2006). One of these instruments, the Fourier-transform ion cyclotron-resonance mass spectrometer (FTICR), provides ultra-high-mass accuracy (below 1 ppm) and the highest mass resolution (more than 1,000,000) (Han et al., 2008). Moreover, using direct infusion coupled to ultra-high-resolution mass spectrometry, metabolites are analyzed in a high-throughput way, providing a rapid analysis of complex metabolite samples, and eliminating the time-consuming separation by liquid chromatography (LC) (Kirwan et al., 2014).

In addition to high mass accuracy instruments, efficient sample extraction methodologies are a priority in metabolomics. These are especially critical when working with plant material, where caution must be taken during harvesting, grinding and metabolite extraction, to avoid consequences in the accuracy of results (Kim and Verpoorte, 2010).

Here we present an efficient metabolite extraction protocol for grapevine leaves, suitable for the characterization of the *V. vinifera* metabolome by direct infusion Fourier-transform ion cyclotron-resonance mass spectrometry (DI-FTICR-MS).

3. Materials and methods

3.1. Plant material

V. vinifera cv Pinot noir young leaves were harvested from five different plants (three biological replicates were considered), at the Portuguese Grapevine Germplasm Bank at INIA-Estação Vitivinícola Nacional (Dois Portos), immediately frozen in liquid nitrogen and stored at -80º C. Leaves were ground in liquid nitrogen and used for metabolite extraction.

3.2. Metabolite extraction

Metabolite extraction from grapevine leaves was performed using different solvent systems coupled to solid phase extraction (SPE) fractionation. We used the mixture 40% methanol (LC-MS grade, Merck)/40% chloroform (Sigma Aldrich)/20% water (v/v/v) as previously described for grapes (Theodoridis et al., 2012), but the ratio was 0.1 g of grinded leaves to 1 ml of solvent. Samples were vortexed for 1 min and maintained in an orbital shaker for 15 min at room temperature. Samples were centrifuged at 1,000*g* for 10 min for phase separation: the lower chloroform fraction and the upper aqueous/methanol fraction. The chloroform fraction (C) was further centrifuged for 5 min at 10,000*g* to remove debris and lyophilized at -40ºC. The aqueous/methanol layer was further processed by SPE using Merck LiChrolut RP-18 columns, pre-equilibrated with methanol. Metabolite fractions were collected by vacuum through sequential elution with 1 ml of water (W), methanol (M) and acetonitrile (A, LC-MS grade, Merck). The water fraction was lyophilized at -40ºC, while both methanol and acetonitrile fractions were evaporated under a nitrogen stream. A workflow of the experimental procedure is shown in Fig. II. 1.

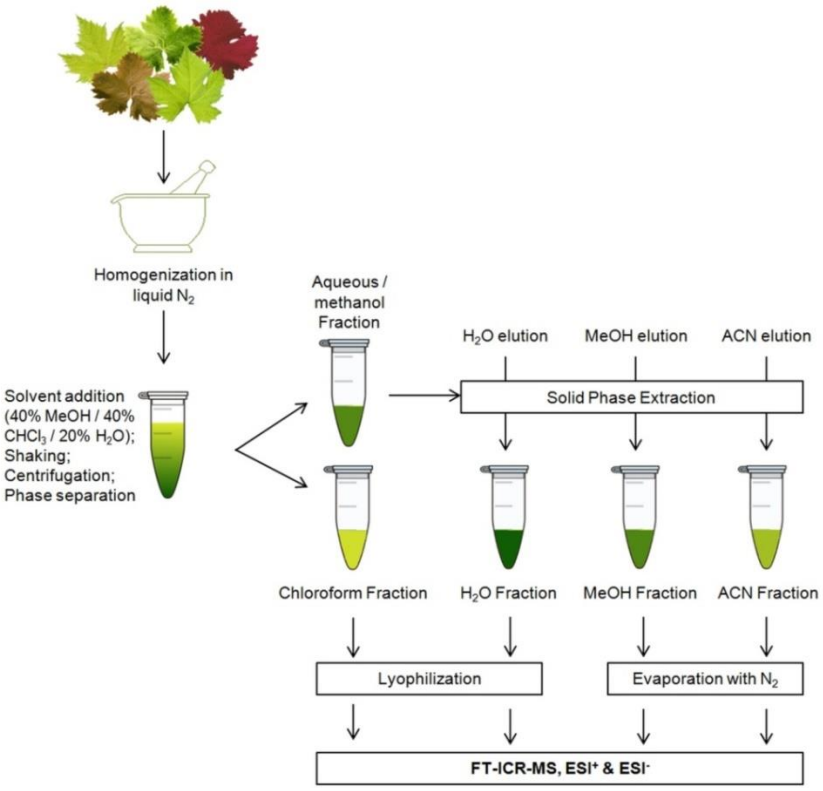

Fig. II. 1 - Experimental procedure for metabolite extraction from grapevine leaves compatible with FTICR-based metabolomics.

3.3. Metabolite analysis by FTICR-MS

W and C fractions were reconstituted in methanol/water (1:1), while M and A fractions were suspended in the respective pure solvent. For the analysis of metabolites, all fractions were diluted 1000-fold in the appropriate solvent: M and A fractions were diluted in the same solvent for positive- (ESI^+) and negative-ion (ESI^-) mode analysis; W and C fractions were diluted in methanol for ESI^+ or in methanol/water (1:1) for ESI^-. The standard leucine enkephalin (YGGFL, Sigma Aldrich) was added to all samples at a concentration of 0.5 µg/mL, and was used as a standard for control and quality assessment of analytical precision ($[M+H]^+$ = 556.276575 Da or $[M-H]^-$ = 554.260925 Da), through the determination of the relative standard peak deviation and internal calibration. For the analysis in ESI^+, formic acid (final concentration 0.1% (v/v), Sigma Aldrich, MS grade) was added to all samples. Extracted metabolites were analysed by direct infusion in the Apex Qe 7-Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR-MS, Brüker Daltonics), with a flow rate of 240 µL h^{-1}. Between each sample run, the ESI source was cleaned with methanol or acetonitrile for 10-15 minutes and the spectrum was collected. Mass spectra were acquired with an acquisition size of 512k, in the mass range between 100 and 1000Da (with a resolution of 130,000 at 400 m/z), and 50 scans were accumulated for each sample. In ESI^+, the nebulizer gas flow rate was set to 2.0 L/min and the dry gas flow rate to 4.0 L/min, at a temperature of 180ºC. The capillary voltage was set to 4500 V and the spray shield voltage was 4000 V. In ESI^-, the nebulizer gas flow rate was 2.5 L/min and the dry gas flow rate was set to 4.0 L/min, at a temperature of 220°C. The capillary voltage was 4300 V and the spray shield voltage was set to 3800 V. In both ionization modes, ions were accumulated in the collision cell for 1.0 s, and a time of flight of 1.0 ms was used prior to their transfer to the ICR cell.

3.4. Data analysis and metabolite identification

Using the Data Analysis 4.1 software package (Brüker Daltonics, Bremen, Germany), the resulting mass spectra were internally calibrated using leucine enkephalin at both ESI modes; external calibration was performed with cyclopamine (*m/z* 412.32100, $[M+H]^+$) in all 4 fractions at ESI^+, whereas at ESI^-, hexadecanoic acid (*m/z* 255.23295, $[M+H]^-$) was used to calibrate M, A and C fractions, while W fraction calibration was calibrated with glutathione (*m/z* 306.07653, $[M+H]^-$). Peak height lists were then exported as ASCII files, setting at a signal-to-noise ratio at 4. The total number of identified ions (peaks) ranged between 1049 and 1346

for ESI^- and 10202 to 11444 for ESI^+ in 3 different biological replicates. The peak lists were combined to a peak matrix with an error of 1.0 ppm, as described by (Lucio et al., 2010), implemented in a Python script based on the Pandas library for data analysis. Peaks with just 1 non-zero intensity (single mass events) were removed from the matrix as well as peaks that were detected in less than 50% of all biological replicates. Overall, 1018 peaks for ESI^- and 6266 peaks for ESI^+ remained after all filtration processes.

For metabolite identification, the mass list was submitted to the MassTrix 3 server [http://masstrix3.helmholtz-muenchen.de/masstrix3/, (Suhre and Schmitt-Kopplin, 2008)] server selecting *V. vinifera* as organism, considering possible adducts M+H and M+Na for ESI^+, and M-H and M+Cl for ESI^- data, with a maximum error acceptance of 3 ppm. A total of 221 masses were annotated for ESI^- and 1366 for ESI^+. A manual curation for compounds with biological role was done by searching the annotated metabolites in the public databases PubChem [http://pubchem.ncbi.nlm.nih.gov/, (Wang et al., 2009)], KNApSAcK [http://kanaya.naist.jp/KNApSAcK/, (Afendi et al., 2012)], KEGG [Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/kegg2.html, (Kanehisa and Goto, 2000)], Lipid Maps [http://www.lipidmaps.org/, (Fahy et al., 2007)] and Metabolomics workbench [http://www.metabolomicsworkbench.org/, (Sud et al., 2015)].

4. Results and discussion

The analysis of unknown metabolites and the biological interpretation of their relationships represent a very important basis for the profiling of unique metabolic systems and the comparison of such profiles in different phenotypes. To ensure meaningful results and high data quality it is important to have a thorough experimental design and an efficient extraction protocol, specifically designed to be used with an accurate analytical technique.

There is no doubt that mass spectrometry (MS) in metabolomics has facilitated the simultaneous detection and quantification of a large number of metabolites within a large dynamic range. Additionally, it provides structural information through fragmentation experiments in the absence of commercially available standards (Becker et al., 2013). Within the several MS instruments available, FTICR is the most powerful tool to fingerprint complex samples, due to its extremely high resolution and high mass accuracy, often better than 1 ppm (Brown et al., 2005; Fiehn, 2002), having therefore a huge potential in the screening of

different samples [as discussed in (Fiehn, 2002; Villas-Bôas et al., 2005)]. However, there are very few studies that use FT-MS in plant metabolomics (Aharoni et al., 2002; Becker et al., 2013; Marques et al., 2016; Motohashi et al., 2015; Oikawa et al., 2006). Most of these studies are based on the analysis of a single extract from the plant material, either using a single solvent, normally methanol or acetonitrile, or a mixture of solvents, either methanol/water or methanol/water/chloroform. The later has several advantages in metabolite extraction yield and reproducibility, by allowing the simultaneous extraction of polar and non-polar metabolites (Wu et al., 2008). In addition, chloroform contributes to protein denaturation, therefore preventing the occurrence of biochemical reactions during the extraction step (Villas-Bôas et al., 2007).

With the goal of maximizing the number of metabolites identified from a single biological sample, particularly from plant secondary metabolism, and to take advantage of the high-resolution and high-mass accuracy of the FTICR, we developed an improved and efficient metabolite extraction method. We used *V. vinifera* cv Pinot noir leaves and started with a mixture of methanol/chloroform/water, as previously described for grapes (Theodoridis et al., 2012). Then, a sequential extraction using solid phase fractionation was followed, and metabolites were eluted with water, methanol and acetonitrile. In total, we obtained four fractions (chloroform, water, methanol and acetonitrile), all analysed by direct infusion-FTICR (DI-FTICR), using electrospray ionization (ESI) in both positive and negative modes. Direct infusion coupled to ultra-high resolution MS provides a rapid analysis of complex metabolite mixtures, eliminating the chromatographic separation, which can be very time-consuming (Kirwan et al., 2014). Indeed, the LC step in Theodoridis *et al.* method increases in 60 minutes each analysis (Theodoridis et al., 2012). On the other hand, in this study, using the same solvent mixture of 40% chloroform/40% methanol/20% water (v/v/v), about 4500 peaks were obtained (Theodoridis et al., 2012), whereas using our workflow the number of identified ions (peaks) reached over 11000. We identified 719 unique masses in leaves of *V. vinifera* cv Pinot noir using DI-FTICR, corresponding to 1383 putative metabolites (since the same mass value can be attributed to more than one compound, particularly isomers), excluding drugs and pesticides. In a previous work using grapevine leaves, only 96 were identified in acetone and butanol extracts by GC-MS (Batovska et al., 2009). More recently, methanol extracts from *V. vinifera* leaves were analysed by FTICR and 40 compounds were detected (Becker et al., 2013). In our work, we identified 158 unique masses only in the methanol fraction in both ionization modes, highlighting the importance of the extraction method for FTICR in non-targeted metabolomics. In potato tubers (*Solanum tuberosum*), the use of FTICR allowed the

identification of at least 152 different compounds only in mitochondria (Marques et al., 2016), whereas in full potato extracts around 150 were detected by GC-MS (Roessner et al., 2000).

In order to increase metabolome coverage, each *V. vinifera* fraction was analyzed in ESI^+ and ESI^-. We identified 144 unique masses by ESI^- and 634 by ESI^+ (Fig. II. 2, A and B, respectively), with only 59 masses common to both ionization modes. In ESI^-, most of the compounds identified were extracted with water and methanol. In the ESI^- mode, compounds as carboxylic acids (e.g citric acid cycle) are preferentially identified (Marques et al., 2016). In *V. vinifera* we found both malate (*m/z* 133.01403, $[M-H]^-$) and citrate and/or isocitrate (*m/z* 191.01990, $[M-H]^-$). Other carboxylic acids, particularly from the glucuronic acid pathway, were also found in our extracts exclusively in the ESI^- analysis, including glucaric acid (*m/z* 209.03011, $[M-H]^-$), glucuronic acid (*m/z* 193.03549, $[M-H]^-$), gluconic acid (*m/z* 195.05114, $[M-H]^-$) and ascorbic acid (*m/z* 175.02494, $[M-H]^-$). Several flavonoids were also identified in negative ionization mode, but most of them were also present in the ESI^+ analysis. When combining the information from both ionization modes, we increase the certainty of the presence of this subclass of metabolites, even if they are in minority in the total extract (Cuyckens and Claeys, 2004). Examples include the flavonoids quercetin (*m/z* 447.09278, $[M-H]^-$ and *m/z* 449.10824, $[M+H]^+$), quercetin 3-*O*-glucoside (*m/z* 463.08800, $[M-H]^-$ and *m/z* 465.10312, $[M+H]^+$), and kaempferol 3-*O*-beta-D-glucosylgalactoside (*m/z* 609.14538, $[M-H]^-$ and *m/z* 633.14392, $[M+Na]^+$), among others. A highly relevant phenolic compound, caffeic acid (*m/z* 179.03516, $[M-H]^-$), and its derivative caffeic acid 3-glucoside (*m/z* 341.08732, $[M-H]^-$), which confer resistance to pathogenic fungi in *V. vinifera* (Figueiredo et al., 2008), were exclusively detected in ESI^-.

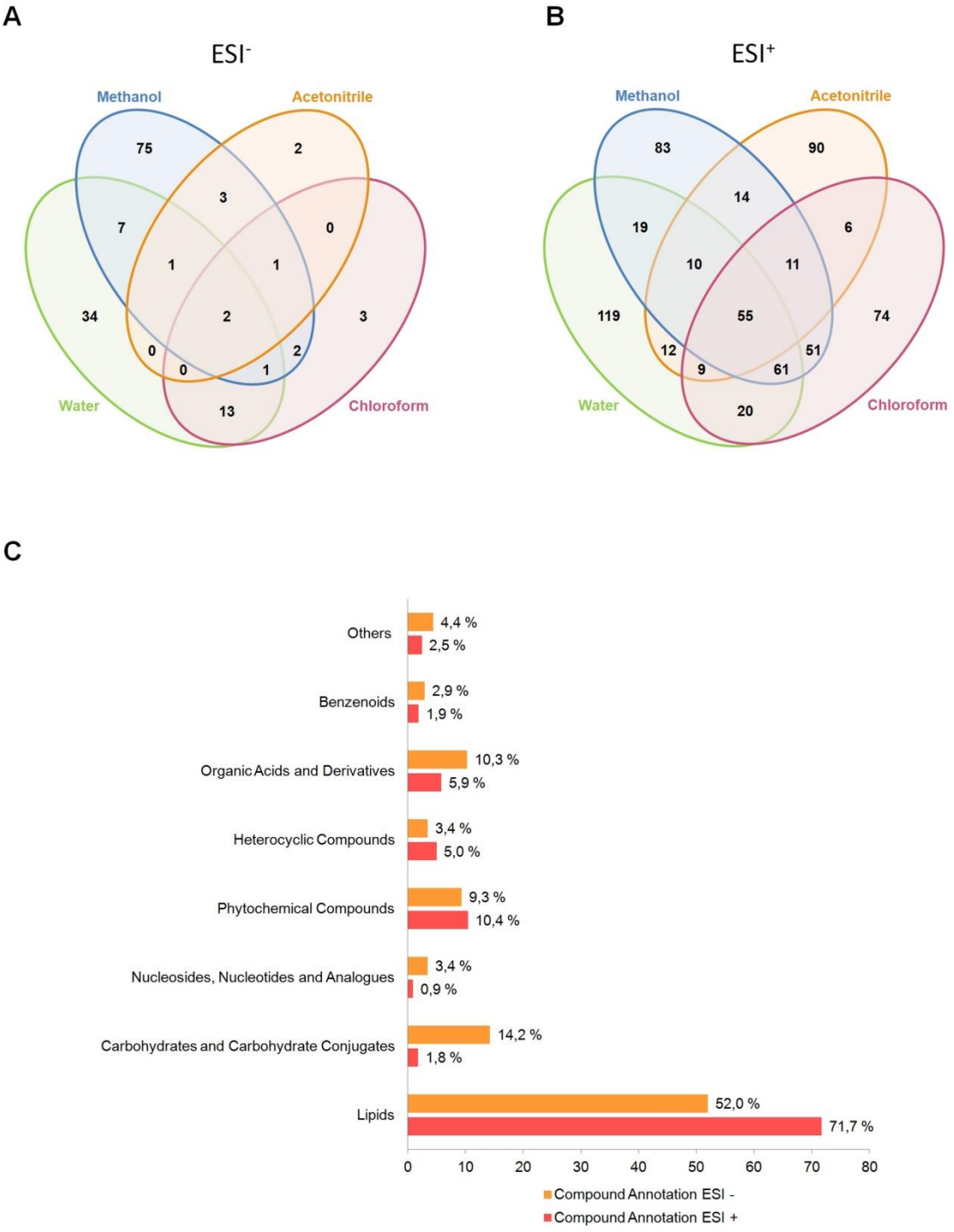

Fig. II. 2 - Grapevine metabolite count and annotation. Four-way Venn diagram summarizing the number of shared metabolites in each fraction in positive (A) and negative (B) ionization modes; compound annotation by major classes, in both ionization modes (C).

It is clear that most of the compounds were identified in the positive ionization mode. In this mode, 55 compounds were common to all fractions, being the water and methanol fractions the ones with most compounds, 305 and 304, respectively, followed by the

chloroform fraction where 287 compounds were identified. In fact, water, methanol and chloroform are the most commonly used solvents in metabolomics (Villas-Bôas et al., 2007). However, 90 unique masses were detected exclusively in the acetonitrile fraction using ESI^+ (Fig. II. 2B). Among the detected compounds, we highlight the alkaloid valeroidine (*m/z* 242.17446, $[M+H]^+$), previously identified in barley as a resistance-related constitutive metabolite in the defence against *Fusarium graminearum* (Bollina et al., 2011). Our results demonstrate that the extraction method that we developed increases the number of extracted metabolites, allowing higher metabolome coverage.

Concerning compound annotation, the detected metabolites were divided in eight different major classes: lipids; carbohydrates and carbohydrates conjugates; nucleosides, nucleotides and analogues; phytochemical compounds; heterocyclic compounds; organic acids and derivatives; benzenoids; and others (compounds with unknown annotation and organonitrogen, organooxygen and organophosphorus compounds), (Fig. II. 2C). The most represented class in *V. vinifera* cv Pinot noir is the Lipids class, defining more than half of the identified compounds, both in positive (71.7 %) and negative (52.0 %) ionization modes. This is not surprising, since we used organic solvents for metabolite extraction, and in fact lipidomics is a major sub-area inside metabolomics (Griffiths et al., 2011). Phytochemical compounds and organic acids (and derivatives) correspond to 10.4% and 5.9% of the total identified metabolites in positive ionization mode, and 9.3% and 10.3% in negative ion mode, respectively.

Concerning metabolite intracellular concentration, we detected not only compounds present in higher levels, but also those found in lower amounts in plants. Among the metabolites present in high levels in plants (Roessner et al., 2007), we identified in *V. vinifera* malate (*m/z* 133.01403, $[M-H]^-$), citrate (*m/z* 191.01990, $[M-H]^-$), sucrose (*m/z* 341.10899, $[M-H]^-$) and the hexoses glucose and/or fructose, and/or galactose (*m/z* 179.05621, $[M-H]^-$), and derivatives. Regarding compounds present in very low concentrations, which is the case for phytohormones, we were able to identify the jasmonic acid derivatives methyljasmonate (*m/z* 247.12980, $[M+Na]^+$) and dihydrojasmonic acid (*m/z* 235.13018, $[M+Na]^+$), acetylsalicylic acid (*m/z* 179.03516, $[M-H]^-$), abscisic acid (*m/z* 265.14381, $[M+H]^+$), the gibberellins A20 (*m/z* 355.15096, $[M+Na]^+$) and allogibberic acid (*m/z* 307.12989, $[M+Na]^+$) and brassinolide (*m/z* 503.33533, $[M+Na]^+$). These results demonstrate that the proposed extraction method was able to extract a wide range of compounds, even those present in low amounts.

In our study, we were also able to detect several pesticides in grapevine leaves. These

compounds were manually curated and excluded from the metabolome annotation. However, for vine leaves production and commercialization, we believe that quality assessment is important and the identification of pesticides is highly relevant for producers to authenticate the quality of the leaves. We were able to detect pesticides such as the insecticide fenthion (*m/z* 312.98909, $[M+Cl]^-$) and the herbicides bromacil (*m/z* 283.00551, $[M+Na]^+$) and terbacil (*m/z* 217.07368, $[M+H]^+$), which are widely used in vineyards to control parasites, insects and fungi. These compounds have serious negative effects in our health, not only by environmental contamination and accidental or intentional poisonings (Prüss-Üstün and Corvalán, 2006), but also by their presence in processed products like grapes and wine (Cabras and Angioni, 2000). Hence, the development of new techniques able to detect pesticides in food products are much needed and the method described here may be a good starting point.

5. Concluding remarks

In conclusion, we developed a metabolite extraction methodology suitable to use with DI-FTICR analysis for untargeted metabolomics. With our extraction protocol, we increased the extraction of polar and non-polar compounds, covering all major classes found in plants. We were able to identify 719 unique masses and also some pesticides in *V. vinifera* cv Pinot noir leaves.

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Chapter III

Metabolic profiling of *Vitis vinifera* cultivar Pinot Noir leaves by FTICR-MS

Maia, M., Marques, A.P., Ferreira, A.E.N., Sebastiana, M., Monteiro, F., Ponces Freire, A., Cordeiro, C., Figueiredo, A., Sousa Silva, M. (2016) Metabolic profiling of *Vitis vinifera* cultivar Pinot Noir leaves by FTICR-MS. *Food Chemistry (submitted)*

Manuscript submitted to *Food Chemistry*

1. Summary

Grapevine (*Vitis vinifera* L.) leaves are a by-product of the vine industry already included in the human diet in several countries of the Mediterranean Basin. Indeed, they contain a wide range of phenolic compounds that confer them high nutritional value, taste and health benefits. In this work, the metabolome of *Vitis vinifera* Pinot noir grapevine leaves was analysed by direct infusion Fourier transform ion cyclotron mass spectrometry (DI-FTICR-MS). Compounds were annotated and divided into major metabolic classes. The lipids class was the most represented accounting for 67% of the annotated metabolome, followed by phytochemical compounds (13%), organic acids (7%) and carbohydrates (3%). Interestingly, within the phenolic compounds class, an acylated anthocyanin [peonidin 3-(6''-acetylglucoside)] was identified for the first time in Pinot noir leaves. These acylated pigments combine their potent antioxidant properties with an increased stability. Additionally, thirteen of the mostly used pesticides were also identified, highlighting the need to identify these compounds in grapevine leaves for quality assessment prior to commercialisation.

2. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important fruit crops worldwide with almost 7375 kha of cultivated area in 2014 (International Organisation of Vine and Wine (OIV), 2015). Around 71% of the production is used for wine, 27% as fresh fruit, and 2% as dried fruit (raisins). Apart from the wine and grape production, in some countries like Turkey (Koşar et al., 2007), Saudi Arabia, Greece, Bulgaria, Romania and Vietnam, many grapevine varieties are grown specifically for the consumption of both fresh and brined green leaves (Rizzuti et al., 2013). Fresh leaf extracts have been used in the treatment of bleeding, high blood pressure, inflammation, diabetes, pain and diarrhea (Ali et al., 2010; Ledesma-Escobar and Luque de Castro, 2015), due to their composition in phenols, mainly flavonols with high antioxidant capacity, and anthocyanins (Fernandes et al., 2013; Koşar et al., 2007). In fact, these compounds have been extracted from grapevine leaves for the development of functional food supplements (Monagas et al., 2006). With an increasing demand for grapevine leaves, top European grapevine producers like Italy are considering these products as important for their economy (FAO Statistical Yearbook, 2014; International Organisation of Vine and Wine (OIV), 2015; Rizzuti et al., 2013).Having in mind the potential of grapevine leaves as a food product, it is important to have the most complete knowledge on metabolite composition, covering all

major classes of compounds. On the other hand, considering the amount of chemical products leaves are exposed to, it is quite relevant to develop accurate detection techniques in order to assure that leaves are safe for consumption. Most studies at the metabolite level in plants for human consumption are based on targeted approaches, focusing on a certain class of compounds, particularly aminoacids, phenolic compounds and fatty acids (Harb et al., 2015; Liu et al., 2014). These studies were performed using one or a combination of analytical techniques like gas chromatography (GC), high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and GC or LC coupled to mass spectrometry (MS), without achieving a maximum metabolome coverage (Ali et al., 2012, 2009, Batovska et al., 2009, 2008; Figueiredo et al., 2008; Harb et al., 2015; Jorge et al., 2015; Liu et al., 2014; Rizzuti et al., 2013). Recent improvements have been made in order to have maximum metabolome coverage. Direct infusion Fourier transform ion cyclotron mass spectrometry (DI-FTICR-MS) presents high-resolution (more than 1,000,000) and ultra-high-mass accuracy (below 1 ppm) (Han et al., 2008) of each analyte in a compound mixture, without requiring any chromatographic separation step. Recently, our group has developed an efficient metabolite extraction protocol for grapevine leaves and identified 719 unique masses, corresponding to over 1300 putative metabolites (Maia et al., 2016). We were also able to detect several pesticides, such as fenthion, bromacil and terbacil, broadly used for parasite, insect and fungi control in grapevine. As the cultivated *Vitis vinifera*, is susceptible to several pathogens, the current strategy for disease control is the application of powerful fungicides and pesticides, which can remain in leaves for a long time. For example, in grapes the dissipation time for fungicides can reach 30 days until they are considered safe for consumption (Banerjee et al., 2008). Thus, it is crucial to have accurate methods for quality assessment of leaf composition.

In this study, we report, for the first time, the metabolic composition of *Vitis vinifera* cv. Pinot noir leaves by DI-FTICR-MS. We identified a total of 1855 unique metabolic entities using both ionization modes. The compounds were annotated and classified into eight metabolic classes (lipids, carbohydrates and conjugates, nucleic acids, phytochemical compounds, heterocyclic compounds, organic acids and derivatives, benzenoids, and others). A metabolic pathway analysis was also done to identify the main metabolic processes, particularly of the phenolic compound group. We also identified 13 of the mostly used pesticides.

3. Materials and methods

3.1. Plant Material

Young grapevine leaves from V*itis vinifera* cv. Pinot noir were collected at the Portuguese Grapevine Germplasm Bank at INIA - Estação Vitivinícola Nacional (Dois Portos). The leaves were harvested (third to fifth insertion from the apex) from 5 different plants, immediately frozen in liquid nitrogen and stored at -80°C. Three biological replicates were collected.

3.2. Metabolite extraction

Sample preparation and metabolite extraction were performed following the protocol previously described by Maia et al. 2016 (Maia et al., 2016). Briefly, 100mg of grinded *Vitis vinifera* cv Pinot noir leaves were added to 1mL of solvent mixture containing methanol, chloroform and water (2:2:1). Samples were submitted to a multistep extraction protocol that includes vortexing and centrifugation to obtain a chloroform fraction and an aqueous-methanol fraction. The chloroform fraction was collected, re-centrifuged and lyophilized at -40°C. The aqueous-methanol fraction was further submitted to solid-phase extraction with Merk LiChrolut RP-18columns and the metabolites were sequentially eluted (twice) with 1mL of water, methanol and acetonitrile. The flow-through fractions were collected by vacuum. The water fraction was lyophilized at -40°C, while both methanol and acetonitrile fractions were evaporated with liquid nitrogen. Water and chloroform fractions were solubilised in a water and methanol solution (1:1), whereas methanol and acetonitrile fractions were reconstituted in the respective pure solvents.

3.3. DI- FT-ICR-MS untargeted metabolomic analysis

Each metabolite fraction was diluted 1:1000 with an appropriate solvent, depending on the electrospray ionization (ESI) mode, as described (Maia et al., 2016). Formic acid (0.1% (v/v), Sigma Aldrich, MS grade) was added to all ESI^+ samples. For quality assessment of analytical precision and internal calibration, the peptide leucine encephalin (YGGFL, Sigma Aldrich) was added to all samples (0.5 µg/mL). For this standard, the following molecular masses were considered for ESI^+ and ESI^- respectively: $[M+H]^+$ = 556.276575 Da and $[M-H]^-$ =

554.2620221 Da. Metabolite analysis was performed by direct infusion in the Apex Qe 7-Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR-MS, Brüker Daltonics), either operating in positive or negative ion mode. Mass spectra were acquired with an acquisition size of 512k, in the mass range between 97 and 1000 m/z. Other proceeding specifications and equipment parameters were previously described (Maia et al., 2016).

3.4. Data analysis of FT-ICR-MS spectra

Mass spectra were analysed using the software package Data Analysis 4.1 (Brüker Daltonics, Bremen, Germany). Internal calibration was performed with the leucine enkephalin standard, both in positive and negative ion modes. Mass peak lists were exported from Data Analysis 4.1 as ASCII files, with a signal-to-noise ratio of 4, without isotopic deconvolution. A peak matrix was generated by the alignment of the mass peak list with an error of 1.0 ppm (as described in Lucio et al., 2010), using a Python based script (Maia et al., 2016). Only masses detected in 2 or 3 biological replicates were considered. The final mass list was uploaded to MassTRIX 3 server in February of 2016 (http://masstrix.org**,** Suhre and Schmitt-Kopplin, 2008) and the following parameters were considered: scan mode was either positive or negative ionization; the adducts considered were M+H, M+K and M+Na for ESI^+ data and M-H and M+Cl for ESI^- data; the maximum error acceptance was 3 ppm; *Vitis vinifera* was the selected organism and the search was performed in the "KEGG/HMDB/LipidMaps without isotopes" database. Metabolite annotation and curation were performed manually (February 2016) using public databases: Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/kegg2.html, Kanehisa and Goto, 2000); KNApSAcK (http://kanaya.naist.jp/KNApSAcK/, Afendi et al., 2012); Lipid Maps (http://www.lipidmaps.org/, Fahy et al., 2007); PubChem (http://pubchem.ncbi.nlm.nih.gov/, Wang et al., 2009); Metabolomics workbench (http://www.metabolomicsworkbench.org/, Sud et al., 2015) and Human Metabolome Database (HMDB) (http://www.hmdb.ca/, Wishart et al., 2007).

3.5. Metabolic pathway analysis

Lipid Maps and HMDB identifiers were converted to KEGG IDs using The Chemical Translation Service (CTS) (http://cts.fiehnlab.ucdavis.edu/, Wohlgemuth et al., 2010)**.**

Metabolic pathway analysis was performed using the KEGG unique identifiers in the KEGG Mapper – Search&Color Pathway analysis tool against the *Vitis vinifera* pathways (prefix: Vvi).

4. Results and discussion

4.1. Overview of grapevine leaves metabolic analysis

Nowadays there has been a growing search for natural compounds, plant based supplements and organic food products. *Vitis vinifera* leaves are one food product that is standing out (Ali et al., 2010; Karaman and Kocabas, 2001; Monagas et al., 2006; Sat et al., 2002). Besides their traditional role in the treatment of inflammatory disorders, pain, diarrhea, bleeding and high blood pressure, they have been related with the prevention and retardation of neurodegenerative diseases associated with oxidative stress (Dani et al., 2010), hepatoprotective activities (Fleming, 2000; Orhan et al., 2007) and antioxidant effects against liver and kidney alcohol associated diseases (Pari and Suresh, 2008). These properties are due to a great diversity of secondary bioactive metabolites in grapevine leaves that have a beneficial effect in human health like vitamins, especially vitamin C (Sat et al., 2002), phenolic compounds, organic acids and lipids (Ali et al., 2010; Bombardelli and Morazzonni, n.d.; J Mann et al., 2007; Monagas et al., 2005; Oancea and Oprean, 2011; Pandey and Rizvi, 2009; Williamson and Holst, 2008)**.**

To characterize the metabolome of V*itis vinifera* cv. Pinot noir grapevine leaves, we used the improved and efficient metabolite extraction method that we previously developed, coupled with direct infusion FT-ICR-MS (Maia et al., 2016). The four obtained fractions (chloroform, water, methanol and acetonitrile) were analysed both in positive (ESI^+) and negative (ESI^-) ionization modes. Mass spectra of methanol fraction in ESI^+ and ESI^- are respectively represented in figure 1 (Fig. III. 1A and B).

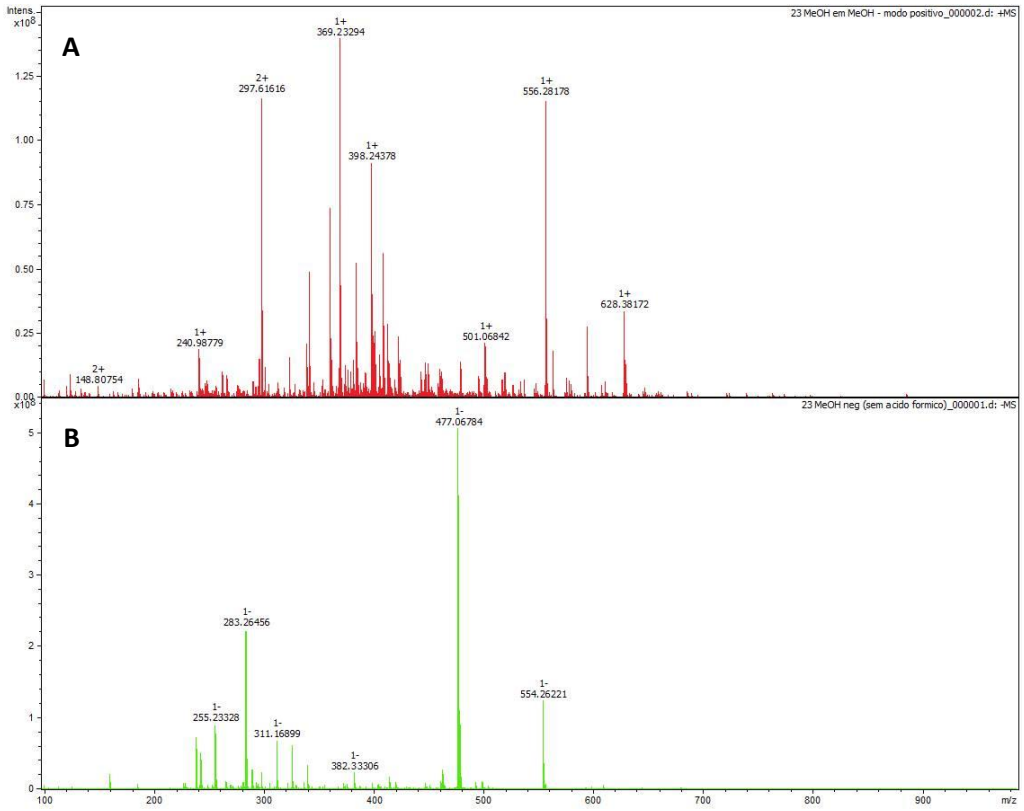

Fig. III. 1 – Methanol fraction representative mass spectra obtained by direct infusion Fourier-transform ion cyclotron-resonance of *Vitis vinifera* cv Pinot noir leaves. A) Positive ionization mode and B) negative ionization mode. The vertical axis represents the intensity of the signal and the horizontal axis represents the mass-to-charge ratio (m/z). Mass spectra were acquired in both ionization modes from 97 to 1000 m/z.

In ESI^+, a total of 6348 ions (peaks) were identified, while in ESI^- 1105 peaks were detected. With the four fractions, we were able to identify a total of 1468 unique masses: 1264 masses in positive ionization mode and 204 masses in negative ionization mode (excluding drugs and pesticides). These compounds were annotated and grouped into eight different metabolite classes (Fig. III. 2).

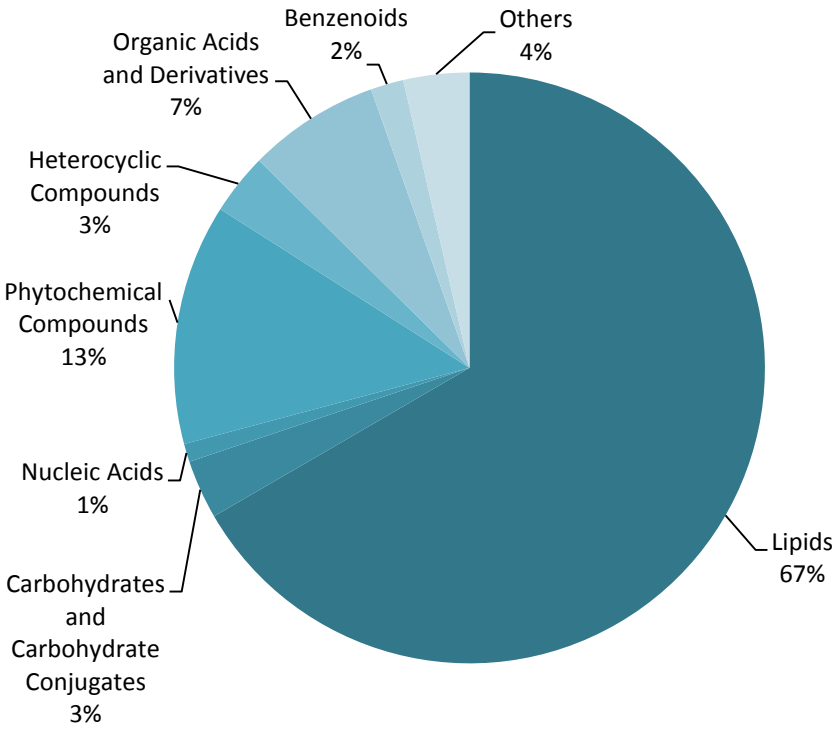

Fig. III. 2 - *Vitis vinifera* cv Pinot noir metabolome annotation. Major classes are represented (according to KEGG, http://www.genome.jp/kegg/kegg2.html).

The most represented metabolic class was lipids (67%), in accordance to our previous grapevine metabolite annotation (Maia et al., 2016), followed by the phytochemical compound class representing 13% of *Vitis vinifera* leaf metabolome. Lipids and phytochemical compounds together with organic acids (7%) and carbohydrates (3%) are the most important plant metabolites, concerning human health, since they are involved in a high amount of processes and as a consequence in the protection of a wide range of diseases (Alabdulkarim et al., 2012; Ali et al., 2010; Dani et al., 2010; Montoro et al., 2005; Orhan et al., 2007; Pari and Suresh, 2008).

4.2. Phenolic compounds

Phenolic compounds are ubiquitous in the plant kingdom. These universally distributed secondary natural metabolites are originated from the shikimate/phenylpropanoid pathway and from the pentose phosphate pathway (Lattanzio, 2013). As major bioactive phytochemicals, these metabolites play an important role in plant defences against environmental stresses, namely pathogen infection, herbivore attack and nutrient deficiency, among others (Braidot et al., 2008; Lattanzio, 2013). Moreover, they have been long

associated to several health beneficial effects, such as the activation of several defence systems, like signalling pathways (e.g. NF-κB), anti-inflammatory activities and the reduction of the risk of chronic diseases (Monagas et al., 2005; Williamson and Holst, 2008).

4.2.1. Flavonoids

Flavonoids are one of the most important metabolites occurring naturally in all *Vitis vinifera* tissues and organs, such as roots, leaves, fruits and seeds (Braidot et al., 2008; Hermosín-Gutiérrez et al., 2011; Lattanzio, 2013)**.** They are one of the most important bioactive phenolic compounds in plant defence as a consequence of their role in response to several biotic and abiotic stress conditions (Braidot et al., 2008; Mattivi et al., 2006; Park and Cha, 2003). Regarding their consumption, flavonoids can be used as a supplement due to their anti-inflammatory, -histaminic, -mutagenic, -carcinogenic and -viral properties (Karakaya and Nehir EL, 1999; Montoro et al., 2005; Park and Cha, 2003; Soleas et al., 2002, 1997). In addition, their anti-oxidant activity in wines, especially white ones, is considered by some authors to be one of the best, in the phenolic compounds group (Burda and Oleszek, 2001; Debeer, 2005; Hermosín-Gutiérrez et al., 2011; Montoro et al., 2005), since they are involved in a wide range of anti-oxidant processes against lipid peroxidation and inhibiting some enzymes involved in the production of radicals, e.g. xanthine oxidase (Montoro et al., 2005)**.**

These bioactive compounds can be grouped in six subclasses: flavonols (kaempferol, quercetin, myricetin and isorhamnetin), flavones (luteolin, apigenin and tangeretin), flavonones (naringenin and hesperetin), flavan-3-ols (catechin, epicatechin and epigallocatechin), anthocyanidins (cyanidin, delphinidin, pelargonidin and malvidin), and isoflavones (genistein, daidzein and glycitein) (Hooper et al., 2008; Lila, 2004; Monagas et al., 2005) and mostly in three possible glycosylated forms (3-O-glucosides, 3-O-galactosides, and 3-O-glucuronides) (Hermosín-Gutiérrez et al., 2011; Mattivi et al., 2006; Talcott and Lee, 2002).

In our study, we identified 121 flavonoids in Pinot Noir leaves. Metabolome pathway analysis using the Search&Color Pathway tool from KEGG allowed us to verify that in flavonoid biosynthesis pathway and flavone and flavonol biosynthesis pathway were identified, respectively, 27 and 14 metabolites (Fig. III. 3).

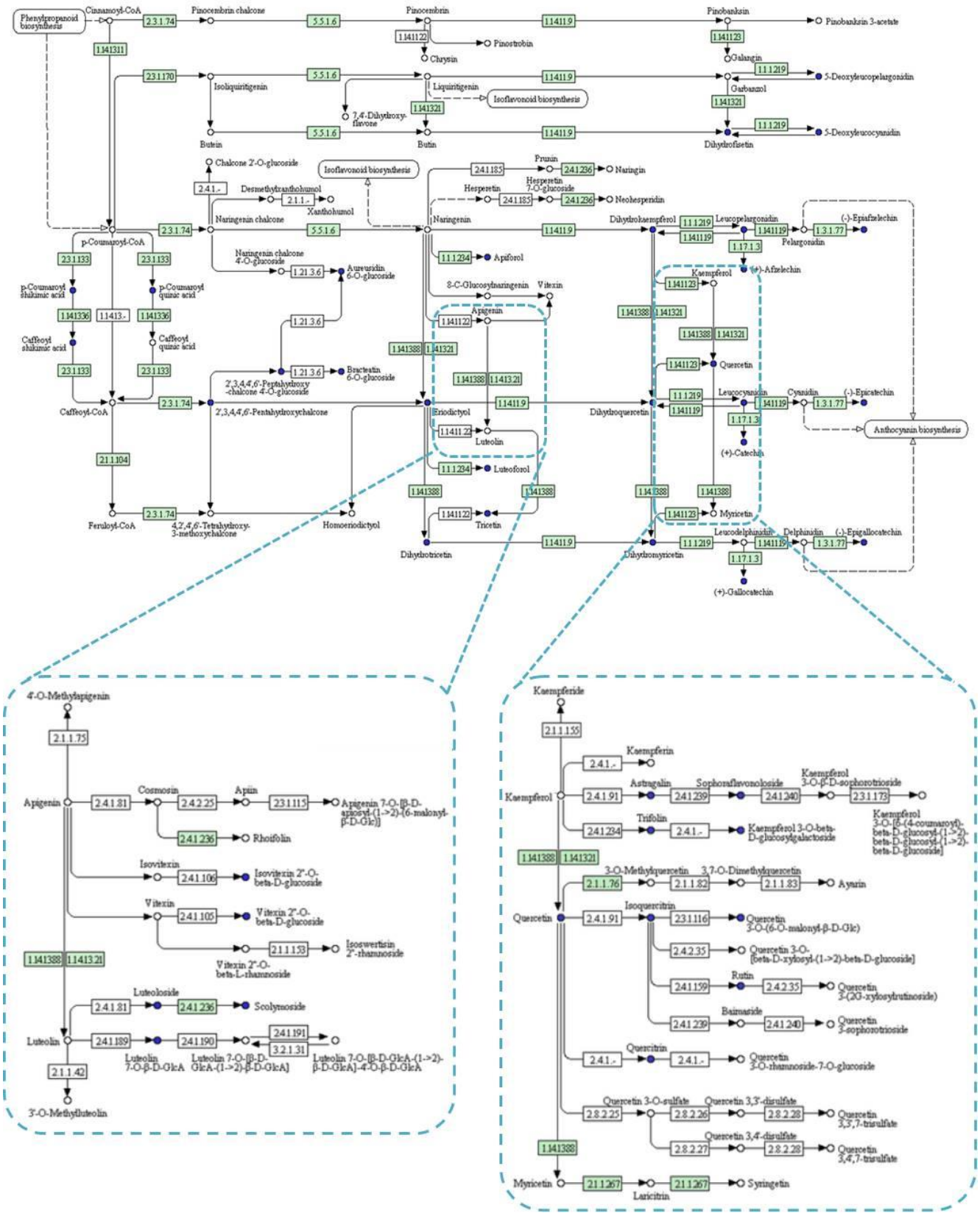

Fig. III. 3 - Flavonoid biosynthesis in *Vitis vinifera*, highlighting the flavone and flavonol biosynthesis pathways (adapted from KEGG). Enzymes shaded in green are present in *Vitis vinifera*. Compounds identified in Pinot Noir leaves by mass spectrometry are highlighted in dark blue.

4.2.1.1. Flavonols

Among the identified flavonols, we highlight the most common 3-O-glycosylated forms and isoforms of flavonols: kaempferol 3-*O*-glucoside (*m/z* 447.09278, $[M-H]^-$ and *m/z* 449.10824, $[M+H]^+$), kaempferol 3-alpha-D-glucoside (*m/z* 447.09278, $[M-H]^-$ and *m/z* 449.10824, $[M+H]^+$), kaempferol 3-*O*-beta-D-galactoside (*m/z* 447.09278, $[M-H]^-$ and *m/z* 449.10824, $[M+H]^+$), kaempferol 3-alpha-D-galactoside (*m/z* 447.09278, $[M-H]^-$ and *m/z* 449.10824 $[M+H]^+$), kaempferol 3-glucuronide (*m/z* 461.07229, $[M-H]^-$ and *m/z* 463.08699 $[M+H]^+$), quercetin 3-*O*-glucoside (*m/z* 463.088, $[M-H]^-$ and *m/z* 465.10312 $[M+H]^+$), quercetin 3-galactoside (*m/z* 463.088, $[M-H]^-$ and *m/z* 465.10312, $[M+H]^+$), isorhamnetin 3-glucuronide (*m/z* 515.07945, $[M+Na]^+$), myricetin 3-glucuronide (*m/z* 493.06164, $[M-H]^-$). Also, a wide range of other glycosylated forms were identified: kaempferol 5-glucoside (*m/z* 447.09278, $[M-H]^-$ and *m/z* 449.10824 $[M+H]^+$), kaempferol 3,7-diglucoside (*m/z* 609.14538, $[M-H]^-$ and *m/z* 633.14392 $[M+Na]^+$), kaempferol 7-galactoside (*m/z* 447.09278, $[M-H]^-$ and *m/z* 449.10824, $[M+H]^+$), kaempferol 3,5-digalactoside (*m/z* 609.14538, $[M-H]^-$ and *m/z* 633.14392, $[M+Na]^+$), kaempferol 7-glucuronide (*m/z* 461.07229 $[M-H]^-$ and *m/z* 463.08699, $[M+H]^+$), kaempferol 5-glucuronide (*m/z* 461.07229, $[M-H]^-$ and *m/z* 463.08699, $[M+H]^+$), quercetin 7-*O*-beta-D-glucoside (*m/z* 463.088, $[M-H]^-$ and *m/z* 465.10312, $[M+H]^+$), quercetin 5-glucoside (*m/z* 463.088, $[M-H]^-$ and *m/z* 465.10312 $[M+H]^+$), quercetin 7-galactoside (*m/z* 463.088, $[M-H]^-$ and *m/z* 465,10312 $[M+H]^+$), quercetin 7-glucuronide (*m/z* 477.06724, $[M-H]^-$ and *m/z* 479.08204, $[M+H]^+$), quercetin 5-glucuronide (*m/z* 477.06724, $[M-H]^-$ and *m/z* 479.08204, $[M+H]^+$) and isorhamnetin 5-galactoside (*m/z* 463.12341, $[M+H]^+$ and *m/z* 485.10631 $[M+Na]^+$). Rutin [quercetin 3-rutinoside (*m/z* 609.14538, $[M-H]^-$ and *m/z* 633.14392, $[M+Na]^+$)] was also detected as well as its derivative quercetin 7-rutinoside (*m/z* 609.14538, $[M-H]^-$ and *m/z* 633.14392 $[M+Na]^+$), detected for the first time in grapevine leaves. Finally, the dihydroflavonol astilbin, that has been reported to be present in grapes and wine (Ali et al., 2010; Guebailia et al., 2006), was also detected in grapevine leaves (*m/z* 449.1095, $[M-H]^-$ and *m/z* 473.10509, $[M+Na]^+$).

4.2.1.2. Anthocyanins

Anthocyanins are a vast group of phytochemical compounds that also belong to the flavonoid family, being responsible for the purple/red to black colour display in fruits, flowers and leaves (Alhamed et al., 2012). Their role in leaves have long been associated to UV

protection, leaf warning, defense against herbivores and pathogens, drought resistance, light backscattering, photoprotection and antioxidant protection (Hatier and Gould, 2008; Lee and Gould, 2002). They are well-accepted in traditional medicine worldwide and it has been reported that anthocyanin isolates or anthocyanin-rich mixtures of bioflavonoids have a wide range of benefits, e.g. in prevention of lipid peroxidation, protection of DNA damage, anti-inflammatory activity, capture of free radicals such as hydroxyl radical ($HO^{\cdot}$), superoxide radical (O_2^{-}), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2), attenuating the nitric oxide production and xanthine oxidase inhibition (Acquaviva et al., 2003; Lazze et al., 2003; Lefevre et al., 2008; Lila, 2004; Oancea and Oprean, 2011; Ramirez-Tortosa et al., 2001; Rossi et al., 2003; Tsuda et al., 1996). In grapes, the anthocyanins cyanidin, delphinidin, petunidin, peonidin and malvidin are normally found in the 3-monoglucoside form or the corresponding acylated form by acetic, coumaric and caffeic acid derivatives (Arozarena et al., 2002; Gao et al., 1997; Mazza and Francis, 1995; Monagas et al., 2006; Pomar et al., 2005). Noteworthy, so far no acylated anthocyanin form has been identified in *Vitis vinifera* cv Pinot noir grapes. In Pinot noir leaves we not only detected peonidin-3-glucoside (*m/z* 486.11376, $[M+Na]^+$), delphinidin 3-*O*-rutinoside (*m/z* 612.16889, $[M+H]^+$) and cyanidin 3,3'-diglucoside (*m/z* 612.16889, $[M+H]^+$) but also other derivatives of these metabolites, like leucocyanidin (*m/z* 305.06694, $[M-H]^-$), 3-deoxyleucocyanidin (*m/z* 289.07193, $[M-H]^-$ and *m/z* 291.08568, $[M+H]^+$), cyanidin 3-galactoside-5-glucoside (*m/z* 612.16889, $[M+H]^+$) and peonidin 3-galactoside (*m/z* 486.11376, $[M+Na]^+$). It is important to note that all of the anthocyanins identified were detected in the water and methanol fractions using the positive ionization mode. Surprisingly, we found peonidin 3-(6''-acetylglucoside) (*m/z* 528.12332, $[M+Na]^+$), an acylated form of peonidin, which is the first acylated anthocyanin detected in Pinot noir leaves. These acylated anthocyanins have increased stability, while retaining their antioxidant properties, being therefore more attractive for use as supplements in commercial food products (Giusti and Wrolstad, 2003).

4.2.2. Stilbenoids

Stilbenoids are another subclass of natural occurring phenolic compounds. Due to their properties, these compounds have been gaining attention and standing out as a natural antioxidant supplement (Ali et al., 2010; Sovak, 2001). Among the stilbenoid group, the most studied metabolite is resveratrol. Although this compound exists in two isomeric forms (*cis* and *trans*), *trans*-resveratrol is the most associated to health benefits regarding cardiovascular

diseases, cancer and anti-inflammatory, -oxidant, -fungal and -microbial processes (Frémont, 2000; Guebailia et al., 2006; Pezet et al., 2003; Vitrac et al., 2005).

Despite stilbenoids, especially resveratrol, being mainly located in grape skin, seeds and stem, they were also detected in *Vitis vinifera* leaves, particularly in response to stress (Wang et al., 2010). In our study, although resveratrol was not identified, we were able to detect the resveratrol dimer ampelopsin (also known as dihydromyricetin, *m/z* 319.046135, $[M-H]^-$) and the resveratrol derivatives dihydroresveratrol (*m/z* 253.082935, $[M+Na]^+$) and 4'-prenyloxyresveratrol (*m/z* 335.12462, $[M+Na]^+$), also known for their, e.g. anti-bacterial and –inflammatory effects, inhibition of apoptosis and hepatoprotective function (Kou and Chen, 2012).

4.3. Lipids

In plants, lipids are a diverse group of compounds which play a wide range of biological activities. Their functions include structural and transport of other compounds in membranes, protection of plants to abiotic and biotic stresses, extracellular and intracellular signalling and storage of energy (Dufourc, 2008; Fahy et al., 2011; Horn and Chapman, 2014; Hou et al., 2016; Laloi et al., 2007; Zauber et al., 2014)**.** Concerning human health, lipids present physiological benefits like protection to Alzheimer's disease (Barberger-Gateau et al., 2011; Morris et al., 2003), obesity (Kunesová et al., 2006) and depression (Stahl et al., 2008). All these different functions are due to an enormous variety of lipid structures. In comparison to other metabolites, lipids have a higher number of complex structures as a result of biochemical transformations that occur during lipid biosynthesis (Fahy et al., 2011). As a consequence of this level of diversity, was developed a correct and suitable classification system. Lipids can be divided into eight major categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides, and each category can be further divided into classes and subclasses (http://www.lipidmaps.org/).

With our methodology, we were able to obtain 1072 lipids in both ionization modes corresponding to 67% of all detected grapevine leaves' metabolites (Fig. III. 2). Within the secondary lipid classes, fatty acids (FA) and prenol lipids (PR) are the most represented with 48% and 16%, respectively (Fig. III. 4).

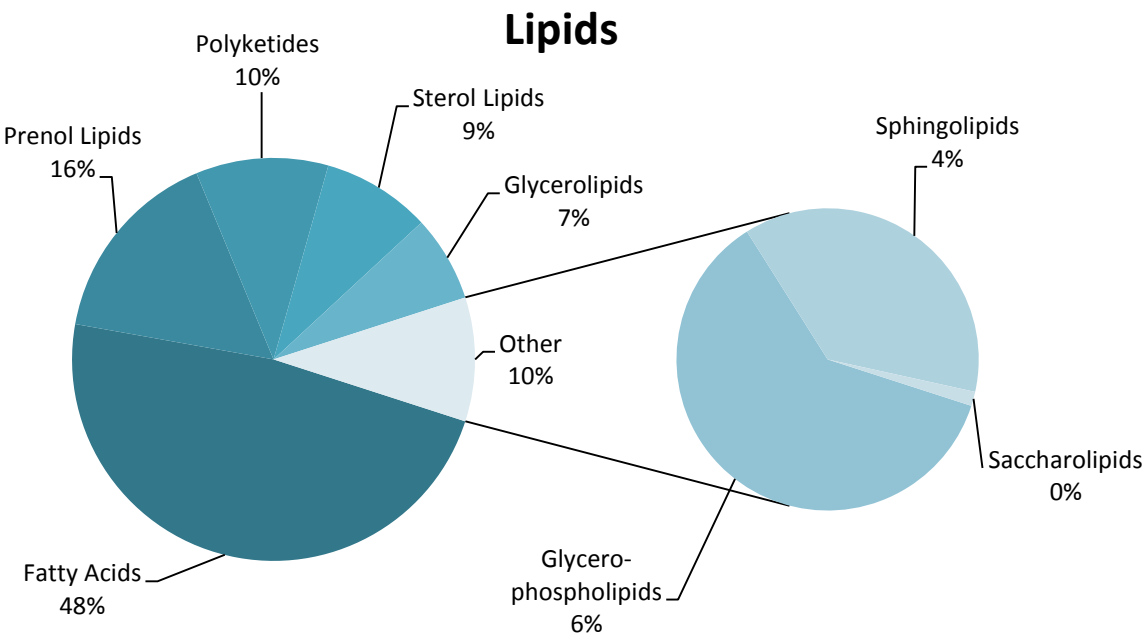

Fig. III. 4 - Lipids secondary class detected in *Vitis vinífera* cv Pinot noir leaves (classification made according to LipidMaps database, http://www.lipidmaps.org/data/classification/LM_classification_exp.php).

Regarding FA and derivatives, these results are not surprising since plants have a large supply of them, synthesizing over 200 different structures of fatty acids with biological functions (Thelen and Ohlrogge, 2002), being the major structural and metabolic constituents of the plant cell. Also, FA play an important role in plants defence to environment and pathogens since they are modulators in transduction signal pathways (Walley et al., 2013). Concerning PR, the fact that it was the second most detected lipid class it is neither surprising since they englobe isoprenoids, the fourth-level lipid class, essentially involved in membrane structure as well as FA. Moreover they are involved in redox mechanisms and are involved in plant defence mechanisms (Enfissi et al., 2004; Osbourn et al., 2011).

4.4. Organic acids and Carbohydrates

Organic acids and derivatives are a large group of compounds which play an important role in the food industry. They are used as acidulants, food additives, flavouring agents and anti-microbial agents in humans' food (Sauer et al., 2008). Moreover, they can also be used in

the production of, e.g. pharmaceuticals and antibiotics (Zeikus et al., 1999). In wine making industry, organic acids are responsible for microbiology and pH control (Ali et al., 2010).

Recently, in leaves of the grapevine cultivar Pinot noir, malic and citric acid were identified, together with organic acids from the glucuronic acid pathway (Maia et al., 2016). In this work, 116 compounds were identified as organic acids and derivatives, corresponding to 7% of Pinot noir leaves' metabolome. Together with carbohydrates and conjugates (3%) represent 10% of the metabolites detected in our experiment.

In grapes, the maturity and cultivar type are affected by the different carbohydrates concentrations. In grapevine leaves and with our methodology we were able not only to detect carbohydrates present in high amounts like glucose (*m/z* 179.05621, $[M-H]^-$) and fructose (*m/z* 179.05621, $[M-H]^-$) but also carbohydrates present in low concentrations like sucrose (*m/z* 341.10897, $[M-H]^-$ and *m/z* 365.10523, $[M+Na]^+$) and galactose (m/z 179.05621, $[M-H]^-$). Carbohydrates have a wide range of positive effects to human health such as an energy source, regulator of appetite, gastrointestinal integrity and function and diabetes type 2 (J. Mann et al., 2007; Stylianopoulos, 2013).

4.5. Pesticides

Vitis vinifera is susceptible to many diseases and, in order to obtain good grapevine quality products, the vineyards must be protected against all kinds of pathogens. Nowadays, the current strategies worldwide include the indispensable application of powerful fungicides and pesticides (Cabras and Angioni, 2000; Gessler et al., 2011). These compounds can remain in leaves for a long time and accurate detection of these compounds must be achieved to guarantee its safety for consumption and commercialization (Banerjee et al., 2008). In our study, we were able to detect 13 of the mostly used chemicals for pest control in plants, like the insecticides quinalphos (*m/z* 297.04595, $[M-H]^-$), fenthion (*m/z* 312.98909, $[M+Cl35]^-$), hydramethylnon (*m/z* 533.154645, $[M+K39]^+$), isofenphos (*m/z* 384.079695, $[M+K39]^+$) and methidathion (*m/z* 302.96938, $[M+H]^+$), and the herbicides terbacil (*m/z* 217.073655, $[M+H]^+$), bromacil (*m/z* 283.00551, $[M+Na]^+$), methyl-bensulfuron (*m/z* 411.09811, $[M+H]^+$), dinoseb (*m/z* 279.03799, $[M+K39]^+$), metsulfuron methyl (*m/z* 382.08246, $[M+H]^+$), pendimethalin (*m/z* 282.14566, $[M+H]^+$), picloram (*m/z* 262.91526, $[M+Na]^+$) and flurochloridone (*m/z* 310.00125, $[M-H]^-$).

5. Conclusions

Vitis vinifera cv Pinot noir leaves were analysed by FT-ICR-MS and 1468 unique masses were identified in both ionization modes. The identified compounds were then annotated into major metabolic classes (lipids, carbohydrates and carbohydrates conjugates, nucleic acids, phytochemical compounds, heterocyclic compounds, organic acids and derivatives, benzenoids and others). Moreover, the most important classes for human health, namely lipids, phytochemical compounds, organic acids and carbohydrates, were the most represented and their health benefits in grapevine leaves were further described. Noteworthy, since we were able to identify thirteen of the most common herbicides and pesticides, it becomes clear that the frequent use of pesticides in vineyards can be dangerous for human consumption and the development of new methodologies to ensure the safe commercialization is a priority.

6. Acknowledgments

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Chapter IV

First clues on metabolic and molecular biomarkers of *Vitis* resistance and susceptibility to *Plasmopara viticola*

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1. Summary

Grapevines display different degrees of resistance and susceptibility to downy mildew disease caused by *Plasmopora viticola*. *Vitis vinifera* L. is the most important fruit crop in the world and its susceptibility to *P. viticola* reflects high financial costs compromising the economy of many countries. Recent studies, have demonstrated that secondary metabolites are involved in grapevine defence response to downy mildew being therefore important biomarkers of resistance. After identification of the relevant metabolic pathways associated with resistance, key enzymes can be highlighted and the genes coding for the selected enzymes can be analysed to evaluate their expression in the innate response. Quantification of the expression of these metabolite coding genes, in grapevine leaves, is a starting point and may be used as a strategy to select resistant plants in breeding programs. In this study, we compared the metabolic composition of ten *Vitis* samples (*Vitis* species and *V. Vinifera* cultivars showing different degrees of resistance towards *P. viticola*) using FT-ICR mass spectrometry. We selected nine distinct masses especially present in the resistant or the susceptible grapevines and identified their metabolic pathways. The expression of several genes coding for enzymes associated with these relevant metabolic pathways, was analysed by qPCR. Quantification of gene expression requires normalization of qPCR data using reference genes with stable expression in the system studied. Hence, a set of eleven genes was evaluated to identify reference genes in the different grapevine leaves. The normalization of the genes of interest was achieved using the three best stable reference genes.

2. Introduction

Downy mildew disease caused by the oomycete *Plasmopara viticola* (Berk. et Curt.) Berl.et de Toni, is one of the most threatening diseases in vineyards (Ali et al., 2010; Figueiredo et al., 2008; Gessler et al., 2011; Polesani et al., 2010a). Nowadays, the most used strategy is the application of powerful pesticides. However, the use of these chemicals is not entirely efficient and not safe for human health since after commercialization they might still be present in grapevine products. Additionally, the extensive fungicide application each growing season has a consequent impact in the economic costs and the environment. As a strategy, pyramiding of resistance genes from American *Vitis* species presenting natural resistance to this pathogen has been used to improve *Vitis vinifera* resistance by breeding programs. The main drawback is time: breeding approaches are quite time-consuming and resource-intensive

until the expression of the trait is observable in the progeny, due to the grapes' long generation cycle and the maintenance requirements of the hybrid cultures. Having this in mind, it is important to uncover biomarkers of resistance (or susceptibility) that will allow a quick and accurate identification of the seedlings that inherited the resistant trait soon after germination. Leaves are the first organ of infection (Gessler et al., 2011), so they represent an excellent starting point to identify secondary metabolites and metabolic pathways related to the downy mildew resistance. Secondary metabolites have been proven to play an important role in plant defences against pathogens, especially *P. viticola*. The study of this pathosystem and knowledge on resistance genes linked to downy mildew has been inferred from several approaches, such as from transcriptional analysis (Figueiredo et al., 2012, 2008; Kortekamp et al., 2008; Malacarne et al., 2011; Polesani et al., 2010b, 2008; Wu et al., 2010) to quantitative trait loci (QTL) and linkage map analysis (Dalbó et al., 2000; Fischer et al., 2004; Jürges et al., 2009; Kortekamp and Zyprian, 2003; Merdinoglu et al., 2003; Peressotti et al., 2010; Welter et al., 2007), and to loci linked to resistance (Bellin et al., 2009; Blasi et al., 2011; Kortekamp et al., 2008; Schwander et al., 2012). Quantitative real-time PCR (qPCR) is currently the most sensitive and reproducible technique for monitoring gene differential expression (Bustin, 2002, 2000; Derveaux et al., 2010). However, qPCR is highly influenced by a number of variables being absolutely necessary an accurate date normalization of genes of interest (GOI) with reference genes (RGs) for qPCR correct measurements of gene expressions.

In this study, we compared ten *Vitis* (wild *Vitis* and *Vitis vinifera*) with different resistance and susceptibility levels to *P. viticola*. Metabolic analysis was performed using Fourier transform ion-cyclotron resonance (FT-ICR) mass spectrometry. Metabolites differentially present in susceptible and resistant cultivars (nine unique masses identified) were used to unfold specific pathways and key enzymes associated with susceptibility or resistance. Transcript levels of selected enzymes were quantified by real-time PCR. This systems biology approach, based on the integration of the metabolic profiling and transcriptomics is the first approach to enlighten the molecular basis of the differences between susceptible and resistant cultivars towards *P. viticola*.

3. Material and Methods

3.1. Plant Material

Six *Vitis* species and four *Vitis vinifera* cultivars, exhibiting different resistance/susceptibility levels towards the oomycete *Plasmopara viticola*, were used in this experiment (Table IV. 1). The resistance of the grapevine species/cultivars towards this pathogen was accessed through bibliographic searches following the classification of Organisation Internationale de la Vigne et du Vin (http://www.oiv.int) and by field behaviour (Estação Vitivinícola Nacional, personal communication Dr. Eiras-Dias). Plants are available at Portuguese Grapevine Germplasm Bank at INIA - Estação Vitivinícola Nacional (Dois Portos). Three leaves (3rd to 5th from the shoot apex) were harvested from 5 different plants (per biological replicate) and immediately frozen in liquid nitrogen. Three biological replicates were analysed.

Table IV. 1 - Type of accession, origin and response to downy mildew of *Vitis* species and *Vitis vinifera* cultivares used in this work.

Code		Type of Accession	Origin	Response to downy mildew
***Vitis* Species**				
LAB	*Vitis labrusca*	Wild species	America	Resistant
RU	*Vitis rupestris*	Wild species	Southern and Western America	Resistant
RT	*Vitis rotundifolia*	Wild species	America	Resistant
RP	*Vitis riparia*	Wild species	North America	Resistant
SYL	*Vitis sylvestris*	Wild species	America	Resistant/Tolerant
CN	*Vitis candicans*	Wild species	Southern America	Resistant
***Vitis vinifera* cultivars**				
PN	Pinot noir	Cultivated grapevine	South Europe	Susceptible
RL	Riesling	Cultivated grapevine	South Europe	Susceptible
TRI	Trincadeira	Cultivated grapevine	South Europe	Susceptible
REG	Regent	Complex hybrid	Breeding	Resistant/Tolerant

3.2. Metabolic extraction and analysis by DI-FT-ICR

Metabolite extraction of each *Vitis* species and cultivars (Table IV. 1) was performed following the protocol developed by Maia et al. 2016 (Maia et al., 2016)**,** with minor modifications. Briefly, metabolite extraction from grapevine leaves was performed using 0.1 g of plant material with 1 ml of 40% methanol (LC-MS grade, Merck)/40% chloroform (Sigma Aldrich)/20% water (v/v/v). Samples were vortexed for 1 min and maintained in an orbital shaker for 15 min at room temperature. Samples were centrifuged at 1000*g* for 10 min for phase separation: the lower chloroform fraction and the upper aqueous/methanol fraction. The aqueous/methanol layer was further processed by SPE using Merck LiChrolut RP-18 columns, pre-equilibrated with methanol. Metabolite fractions were collected by vacuum through elution with 1 ml water and then with 1 ml methanol. Only the methanol fraction was further processed and analysed in this work. Methanol was evaporated under a nitrogen stream and the fraction was frozen at -80°C until further use. Samples were analysed using direct infusion Fourier transform ion cyclotron resonance mass spectrometry (DI-FTICR-MS) in positive (ESI^+) and negative (ESI^-) ionization modes, as previously described (Maia et al., 2016)**.** Mass spectra of each *Vitis* specie/cultivar were processed as described, considering masses only present in more than 2 biological replicates.

3.3. Data analysis with MassTrix

Mass lists from *Vitis* species and *Vitis vinifera* cultivars, both from positive and negative ion modes, were uploaded to MassTrix 3 server (February of 2016) (http://masstrix3.helmholtz-muenchen.de/masstrix3/, Suhre and Schmitt-Kopplin, 2008; Wägele et al., 2012). Each Job ID was saved for further analysis using the MassTrix Job compare tool at the compound level (Wägele et al., 2012). This functionality allows the comparison of all annotated compounds in the different experiments.

3.4. RNA extraction and cDNA synthesis

Total RNA was isolated from leaves of the different *Vitis* species/cultivars using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA), according to the manufacturer's instructions. After extraction, all samples were analysed spectrophotometrically, absorbance ratios at 260/280 and 260/230 nm above 1.8, and in agarose gels, showing well-defined bands

corresponding to the rRNA and absence of nucleic acid degradation. To confirm the absence of contaminating genomic DNA (gDNA), positive and no reverse transcription (RT) controls were used for each candidate gene amplification. DNase treatment (On-Column DNase I Digestion, Sigma-Aldrich) was followed by a careful check for the absence of gDNA through qPCR analysis of a target on the crude RNA (Vandesompele et al., 2002a). Complementary DNA (cDNA) was synthesized from 2.5 µg of total RNA using RevertAid® H Minus Reverse Transcriptase (Fermentas, Ontario, Canada) anchored with Oligo$(dT)_{23}$ primer (Sigma-Aldrich, USA), according to manufacturer's instructions.

3.5. Selection of candidate genes

3.5.1. Reference genes (RG)

Candidate genes (eleven in total) were selected based on their previous description as good qPCR control genes for *Arabidopsis thaliana* (Czechowski et al., 2005) and grapevine (Monteiro et al., 2013; Polesani et al., 2010b; Reid et al., 2006). Nine of the selected genes were previously described as reference genes for grapevine: 60S ribosomal protein L18 (*60S*), small nuclear ribonucleoprotein SmD3 [currently annotated as Tetratricopeptide repeat protein 7B (*TTC7B*)], elongation factor 1-alpha (*EF1α*), ubiquitin-conjugating enzyme (*UBQ*), SAND family protein (*SAND*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), alpha-tubulin 3-chain (α-*TUB*), beta-tubulin 1-chain (β-*TUB*) and Actin (*ACT*). The other two genes were previously described for *Arabidopsis* and grapevine sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) or grape genome browser (http://genomes.cribi.unipd.it/grape/) as being homologous to *Arabidopsis* adaptor protein-2 MU-adaptin (*AP2M*) and Photosystem I P700 chlorophyll a apoprotein A2 (*PsaB*), respectively (Table IV. 2).

3.5.2. Biomarker candidate genes (Genes of interest – GOIs)

Biomarker candidate genes were selected based on biochemical pathways that are relevant for the grapevine defence against *Plasmopara viticola*. Nine metabolites were selected based on previous reports demonstrating: a) they may play an important defence role in grapevine leaves against *P. viticola* (hexadecanoic acid) (Batovska et al., 2008); b) they are differentially accumulated in resistant grapevine varieties after inoculation with *P. viticola*

(caffeic acid, fumaric acid, glutamic acid, myo-inositol, quercetin and quercetin 3-*O*-glucoside) (Ali et al., 2012); and c) they are present exclusively in the resistant or susceptible species/cultivars analysed in the present study (catechin and epicatechin). Biosynthesis or/and degradation enzymes for each metabolite were considered for gene expression analysis. Gene search was performed using data from literature. Genes coding for the enzymes involved in catechin and epicatechin synthesis were previously described in *Vitis* (Bogs, 2005; Gagné et al., 2009), and the remaining coding genes were selected using the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) at NCBI (http://www.ncbi.nlm.nih.gov/) by searching the *Arabidopsis thaliana* homologue genes in the *Vitis vinifera* genome (Table IV. 3).

Table IV. 2 - Candidate Reference Genes primer sequences, amplicon length and qPCR conditions

Gene (NCBI Accession Number)	Primer sequence	Amplicon length (bp)	Ta (°C)	Tm (°C)
60S (XM_002270599.1)	**Fw:** ATCTACCTCAAGCTCCTAGTC **Rev:** CAATCTTGTCCTCCTTTCCT	165	60	79.6
TTC7B (XM_010650160.1)	**Fw:** GCTCTGTTGTTGAAGATGGG **Rev:** GGAAGCAGTTTGTAGCATCAG	156	60	79.9
EF1α (XM_002284888.2)	**Fw:** GAACTGGGTGCTTGATAGGC **Rev:** ACCAAAATATCCGGAGTAAAAGA	164	60	79.8
UBQ (XM_002284161.3)	**Fw:** GAGGGTCGTCAGGATTTGGA **Rev:** GCCCTGCACTTACCATCTTTAAG	75	60	78.9
SAND (XM_002285134.2)	**Fw:** CAACATCCTTTACCCATTGACAGA **Rev:** GCATTTGATCCACTTGCAGATAAG	76	60	79.2
ACT (XM_010659103.1)	**Fw:** ATTCCTCACCATCATCAGCA **Rev:** GACCCCCTCCTACTAAAACT	89	55	77.5
GAPDH (XM_002263109.3)	**Fw:** TCAAGGTCAAGGACTCTAACACC **Rev:** CCAACAACGAACATAGGAGCA	226	60	81.4
PsaB (VIT_209s0002g08330.1)	**Fw:** GGACCCCACTACTCGTCGTATT **Rev:** TCCGGAAGTCCACAGAAAAAT	148	60	76.9
α-*TUB* (XM_002285685.3)	**Fw:** CAGCCAGATCTTCACGAGCTT **Rev:** GTTCTCGCGCATTGACCATA	119	60	79.2
AP2M (XM_002281261.2)	**Fw:** CCTCTCTGGAATGCCTGATTT **Rev:** CTTTAGCAGGACGGGATTTA	89	55	75.0
β-*TUB* (XM_002275270.3)	**Fw:** TGAACCACTTGATCTCTGCGACTA **Rev:** CAGCTTGCGGAGGTCTGAGT	86	60	81.5

bp –base pairs; Ta – Annealing temperature; Tm – melting temperature;

Table IV. 3 - Candidate biomarker genes primer sequence, amplicon length and qPCR conditions

	Metabolite	Enzyme	Abbreviation	EC number	Gene NCBI Accession Number	Primer sequence	Amplicon length (bp)	Ta (°C)	Tm (°C)
1	**Caffeic acid**	Caffeic acid *O*-methyltransferase*	COMT	EC 2.1.1.68	XM_003634113	Fw: GTATGACCCCAACAACTATC Rev: GACCATGGGGAGAACTGA	88	60	78.4
		p-coumarate 3-hydroxylase	C3H	EC 1.14.13.-	XM_002283302	Fw: ACTTCTCCAACCTCCCTTAC Rev: GCCACAGCCCATACATTTAC	162	60	81.4
2	**Catechin**	Leucoanthocyanidin reductase 2	LAR2	EC 1.17.1.3	AJ865334	Fw: TGTAACCGTGGAAGAAGATGA Rev: ATGAAGATGTCGTGAGTGAAG	92	60	80.3
3	**Epicatechin**	Anthocyanidin reductase	ANR	EC 1.3.1.77	BN000166	Fw: ATCAAGCCAGCAATTCAAGGA Rev: CAGCTGCAGAGGATGTCAAA	93	60	76.2
4	**Fumaric acid**	Fumarate hydratase I	FUM1	EC 4.2.1.2	XM_002272997	Fw: TTCCCTACACCCCATTCACA Rev: CGTACATCATCATTTCCCTTC	87	60	76.6
5	**Glutamic acid**	Ferredoxin-dependent Glutamate synthase	Fd-GOGAT	EC 1.4.7.1	XM_002267020	Fw: AGAAGCTAGCGCAGAGTTTG Rev: CATTTCGCCATATCCCCATTA	196	60	79.0
		NADH-dependent Glutamate synthase	NADH-GOGAT	EC 1.4.1.13 EC 1.4.1.14	XM_002267829	Fw: CCGACGGAGGACACTAAAAT Rev: CTCCAGCACAAAAACTCACAA	101	60	74.2
6	**Hexadecanoic acid**	Fatty acyl-ACP thioesterase B	FatB	EC 3.1.2.14 EC 3.1.2.21	XM_002284814	Fw: TCGCAAACCCTAGAAACCAAT Rev: AATGAGGGAAGGAGGAAAATG	112	60	77.4
7	**Myo inositol**	Myo-inositol monophosphatase	IMPL1	EC 3.1.3.25	XM_002276661	Fw: ATCCCAAACGCTACCCAAAAA Rev: TAACAGCTTCCATCACAACCT	119	60	80,6
8	**Quercetin**	Flavonoid 3',5'-hydroxylase	F3'5'H	EC 1.14.13.88	AB213606	Fw: GTGGTGCCGGAGATGTTA Rev: TGCGATGGACGGAATAAAAT	173	56	80.0
9	**Quercetin-3-*O*-glucoside**	UDP-glucose:flavonoid 3-*O*-glucosyltransferase	UFGT	EC 2.4.1.91	AF000372	Fw: AGGGGATGGTAATGGCTGT Rev: ATGGGTGGAGAGTGAGTTAG	151	60	84.8

*Degradation enzyme; Ta – Annealing temperature; Tm – melting temperature

3.5.3. Primers design

Candidate reference and biomarkers genes primers were designed with Primer Express software version 3.0 (Applied Biosystems, Sourceforge, USA). The used parameters were: amplicon length between 80 and 250 bp; size: 20 $\pm$ 2 bp; melting temperature (Tm): 58 $\pm$ 2°C; GC content around 50% (Table IV. 2; Table IV. 3).

3.6. Real time PCR

Quantitative RT-PCR (qPCR) experiments were carried out using Maxima™ SYBR Green qPCR Master Mix (2×) kit (Fermentas, Ontario, Canada) in a StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, USA). A final concentration of 2.5 mM $MgCl_2$ and 0.2 µM of each primer were used in 25 µL volume reactions, together with cDNA as template. To assess the amplification efficiency of each candidate/target gene, identical volumes of all cDNA samples (1 µl) were pooled. The pool was diluted and used to generate a five-point standard curve based on a ten-fold dilution series. Each standard curve was amplified in two independent qPCR runs and each dilution was run in duplicate. Amplification efficiency (E) was calculated from the slope of the standard curve ($E = 10^{(-1/a)} - 1$) where a is the slope of the linear regression model ($y = \log(x) + b$) fitted over log-transformed data of the input cDNA concentration (y) plotted against quantification cycle (Cq) values (x).

Thermal cycling for all genes started with a denaturation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing temperatures (Table IV. 2; Table IV. 3) for 30 s. Each set of reactions included template and no template controls. Non-specific PCR products were analysed by melting curves. Three biological replicates and two technical replicates were used for each sample.

3.7. Determination of gene expression stability

In order to evaluate gene stability, regardless the genotype towards downy mildew infection, all species and cultivars were analysed together. The stability of candidate reference genes was evaluated with three publicly available software tools, GeNorm v. 3.5 (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and the BestKeeper tool (Pfaffl et al., 2004). A comprehensive ranking of the candidate genes was established by

calculating the arithmetic mean of the ranking in each algorithm used, as reported previously (Wang et al., 2012). Each gene was ranked from 1 (most stable) to 11 (least stable). The definition of the optimal number of genes required for normalization was achieved by GeNorm pairwise variation analysis (Tunbridge et al., 2011). Additionally, RefFinder (http://fulxie.0fees.us/) was used as a verification tool of our results.

3.8. Gene expression analysis

The expression of the candidate genes involved in the biosynthesis/degradation pathways of the selected metabolites (Table IV. 3) was further evaluated by qPCR as described. The expression of the candidate genes in *Vitis* species and cultivars, presenting different levels of resistance towards *Plasmopora viticola*, was compared relatively to the *Vitis vinifera* cv Pinot noir expression (susceptible to *P. viticola*). To assess gene expression, relative quantities (RQ) were calculated for both RGs and biomarkers candidate genes by the formula $\mathrm{RQ} = \mathrm{E}^{\Delta \mathrm{Cq}}$, where E represents the amplification efficiency for each gene and $\Delta\mathrm{Cq}$ the difference in the Cq from each target sample and calibrator ($\Delta Cq = Cq_{Control\ samples} - Cq_{Target\ sample}$) (Hellemans et al., 2007). A normalization factor, calculated as the geometric mean of the relative expression of the three best reference genes (selected by the comprehensive ranking after the gene stability analysis of the three applets) (see "*Determination of gene stability*"), was used.

4. Results and discussion

4.1. Metabolic comparison between susceptible and resistant *Vitis* to *Plasmopora viticola*

Grapevine is often affected by a variety of pathogens and if these pathogens are not controlled, the damage can be harsh affecting all vineyards. The degree of susceptibility/resistance depends on the *Vitis* species. In response to pathogen attack, it has been shown that the biosynthesis of secondary metabolites is one of the most important plant defence mechanisms (Bennett and Wallsgrove, 1994). In this study, the metabolic composition of six *Vitis* species and four *Vitis vinifera* cultivars, with different resistance/susceptibility levels towards the oomycete *Plasmopara viticola* (Table IV. 1), was performed using high resolution

mass spectrometry. The purpose was to identify specific compounds in resistant/tolerant or susceptible grapevines towards the identification of biomarkers of resistance (or susceptibility). In this first analysis, we only analysed the methanol fraction since it has been demonstrated (Maia et al. 2016) that the majority of compounds extracted from grapevine leaves are identified in this fraction.

This analysis allowed us to identify a total of nine unique masses that discriminated the resistant from the susceptible grapevines: six in ESI^- and three in ESI^+ (Table IV. 4). In the negative ionization mode, four unique masses were identified in resistant/tolerant grapevines (*Vitis rotundifolia*, *V. riparia*, *V. labrusca*, *V. rupestris*, *V. candicans*, *V. sylvestris* and *Vitis vinifera* cultivar Regent) and only two masses were detected in susceptible grapevines (*Vitis vinifera* cultivares Riesling, Pinot noir and Trincadeira) (Table IV. 4). For ESI^+, the three unique masses were only identified in resistant/tolerant grapevines. Moreover, we verified if the compounds described in literature as associated to grapevine resistance (caffeic acid, fumaric acid, glutamic acid, hexadecanoic acid, myo-inositol, quercetin and quercetin-3-o-glucoside) were also present in our metabolic comparison (Table IV. 4). Fumaric acid and glutamic acid were not detected, but caffeic acid was detected in ESI^-. The remaining compounds were detected in both ionization modes and except from caffeic acid, which is the only compound present in susceptible grapevines (*Vitis vinifera* cultivares Riesling and Pinot noir), the majority of the compounds are present in both resistant and susceptible *Vitis* samples. Although these compounds were not exclusively present in resistant or susceptible groups, we have decided to analyse the constitutive expression of enzyme coding genes related to the biosynthesis of these metabolites (Table IV. 3). From our metabolic comparison, we decide to select the two masses presenting the highest number of isomers: *m/z* 325.0484 and *m/z* 327.0454, corresponding to catechin and epicatechin. These two compounds belong to a particular anthocyanin group, proanthocyanidins, involved in the protection of plants against pathogens (Bogs, 2005).

Table IV. 4 - *Vitis* species and *vinifera* cultivars distinct putative compounds, respective *m/z*, adduct and ionization mode detected. Compounds of interest involved in grapevine defences selected for this study are identified. X represents the presence of the metabolite in each *Vitis* sample.

			***Vitis* species**						***Vitis vinifera* cultivar**				**Compounds of interest involved in defence**
			Resistant					**Tolerant**		**Susceptible**			
Putative compound	**Identified *m/z* by FTICR-MS**	**Ionization mode**	***rotundifolia***	***riparia***	***labrusca***	***rupestris***	***candicans***	***sylvestris***	**Regent**	**Riesling**	**Pinot noir**	**Trincadeira**	
Gallotannin	635.0889, [M-H]-	Neg	X	X		X							
Chicoric acid	473.0725, [M-H]-	Neg	X	X		X							
Coixenolide	589.5193, [M-H]-	Neg		X	X	X	X	X	X				
Pyridoxine 5'-phosphate	284.0099, [M+Cl35]-	Neg		X		X			X				
Catechin or Epicatechin	325.0484, [M+Cl35]-	Neg								X	X	X	
Catechin or Epicatechin	327.0454, [M+Cl37]-	Neg								X	X	X	
N-(1-methyl-2-hydroxy-2-phenyl-ethyl)-5Z,8Z,11Z,14Z-eicosatetraenoyl amine	438.3366, [M+H]+	Pos		X	X		X						
Apiole Or Dillapiole	245.0784, [M+Na]+	Pos			X			X	X				
Uridine	283.0326, [M+K39]+	Pos		X	X			X					
Caffeic acid	179.0349, [M-H]-	Neg								X	X		X (Ali et al., 2012)
Fumaric acid	------	------											X (Ali et al., 2012)
Glutamic acid	------	------											X (Ali et al., 2012)
Hexadecanoic acid	255.2329, [M-H]-	Neg	X	X	X	X	X	X	X	X	X	X	X (Batovska et al., 2009)
	257.2475, [M+H]+	Pos				X					X		
Myo inositol	179.0561, [M-H]-	Neg						X		X			X (Ali et al., 2012)
	203.0526, [M+Na]+	Pos				X			X	X			
Quercetin	301.0353, [M-H]-	Neg		X	X	X	X	X	X	X	X		X (Ali et al., 2012)
	303.0499, [M+H]+	Pos	X	X	X	X	X	X	X	X	X	X	
Quercetin-3-O-glucoside	463.0881, [M-H]-	Neg	X		X		X	X	X	X	X	X	X (Ali et al., 2012)
	465.1027, [M+H]+	Pos	X	X	X	X	X	X	X	X	X	X	

4.2. Selection of reference genes

Quantitative real time polymerase chain reaction (qPCR) is the most sensitive technique for monitoring gene differential expression due to its reproducibility and sensitivity (Bustin, 2002, 2000; Derveaux et al., 2010). Regardless of being an extremely powerful technique regarding sensitivity, specificity and broad quantification range, qPCR is influenced by a number of variables that strongly interfere with its accuracy and reliability (Bustin et al., 2005; Derveaux et al., 2010; Huggett et al., 2005). As a result, qPCR studies require one or more reference genes (RGs) as internal controls for the normalization of raw expression data, allowing the correction of variable starting amounts of RNA and differences in reverse transcription (RT) efficiency, since reference genes are exposed to the same preparation steps as the genes of interest (GOI) (Bustin, 2002; Bustin et al., 2005; Fleige et al., 2006; Huggett et al., 2005). Reference genes must be validated for each experimental condition (Schmittgen and Zakrajsek, 2000) and the geometrical averaging of multiple internal control genes should be used (Vandesompele et al., 2002b). In grapevine, validation of reference genes has been reported for berry development (Reid et al., 2006), abiotic stress (Coito et al., 2012), and biotic stress (Gamm et al., 2011; Selim et al., 2012). However, these cannot be used in our work, where a wide range of *Vitis* species and *V. vinifera* cultivars were analysed. Hence, we have tested eleven candidate reference genes for qPCR normalization of gene expression (Table IV. 2) in different *Vitis* species/cultivars showing different degrees of resistance towards *P. viticola* (Table IV. 1).

4.2.1. Analysis of RG expression stability data

To examine the expression stability of the candidate RGs selected, transcript levels of the eleven candidates were measured by qPCR using gene-specific primer pairs (Table IV. 2). Melting curves of the genes tested were analysed to detect the absence/presence of primer dimer or non-specific PCR products. For all candidate RGs tested, this analysis showed specific gene amplification and no primer dimer formation on the amplicon region, being all genes considered suitable for further analysis.

To assure that any variation between biological replicates was not related to the treatments but intrinsic to the gene itself, the expression stability of the candidate RGs was evaluated by the three statistical algorithms GeNorm, Normfinder and Bestkeeper and verified with the RefFinder tool (Castro et al., 2011; Remans et al., 2008). RefFinder integrates the

currently available major computational programs (GeNorm, Normfinder, BestKeeper, and the comparative ΔCt method) and, based on the rankings from each program, assigns an appropriate weight to an individual gene and calculates the geometric mean of their weights for the overall final ranking. The results were analysed considering all *Vitis* samples (*Vitis* species and *vinifera* cultivares) in the assay.

4.2.2. Statistical analysis of expression stability of the candidate RGs using GeNorm, NormFinder and BestKeeper

GeNorm is based on the pairwise variation of a single reference candidate gene relative to all other genes. GeNorm algorithm calculates a gene expression stability measure (M value) for each gene, based on the average pairwise expression ratio between a gene and each of the other genes being compared in the analysis. So, a gene displaying a low M value presents a low variance in its expression. Ranking order of the most stable to the least stable genes is presented in parenthesis (Table IV. 5). In all the *Vitis* species/cultivars analysed, genes encoding for UBQ and SAND were ranked as the most stable genes presenting the lowest M value (M = 0.859), followed by *GAPDH* (M = 0.990) and EF1α (M = 1.027).

NormFinder is based on a variance estimation approach, which calculates an expression stability value (SV) for each gene analysed. It enables estimation of the overall variation of the reference genes, taking into account intra and intergroup variations of the sample set. According to this algorithm, genes with lowest SV will be top ranked (Andersen et al., 2004). For all *Vitis* samples analysed, *UBQ* was considered as the most stable gene with an SV = 0.552 (Table IV. 5), followed by *AP2M* (SV = 0.744), *GAPDH* (SV = 0.745) and β-*TUB* (SV = 0.766).

The BestKeeper tool calculates standard deviation (SD) based on quantification cycle (Cq) values of all candidate reference genes (Pfaffl et al., 2004). Moreover, BestKeeper compares each reference gene to the BestKeeper Index (BKI) and calculate a Pearson correlation coefficient (r). Higher r values suggest more stable expression. Genes with SD less than 1 and with the highest coefficient of correlation have the highest stability. In this study, BestKeeper analysis considered α-*TUB* and *SAND* as the most stable genes for all *Vitis* samples, with SD values of 0.92 and 1.01, respectively (Table IV. 5). *60S* (SD = 1.07) was the third and *EF1α* (SD = 1.09) was the fourth most stable genes.

Table IV. 5 - Candidate reference genes ranking for all *Vitis* samples calculated by GeNorm, NormFinder and BestKeeper. Genes are ordered by the final ranking.

Genes	GeNorm	NormFinder	BestKeeper		Ranking mean	Final ranking
	M value	SV	SD	*r*		
UBQ (Ubiquitin-conjugating enzyme)	0.859 (1)	0.552 (1)	1.13 (5)	0.90*	2.33	**1**
SAND (SAND family protein)	0.859 (1)	0.791 (6)	1.01 (2)	0.81*	3.00	**2**
EF1α (Elongation factor 1-alpha)	1.027 (4)	0.767 (5)	1.09 (4)	0.85*	4.33	**3**
AP2M (Adaptor protein-2 MU-adaptin)	1.105 (5)	0.744 (2)	1.20 (6)	0.86*	4.33	**3**
GADPH (Glyceraldehyde-3-phosphate dehydrogenase)	0.990 (3)	0.745 (3)	1.31 (8)	0.90*	4.67	**4**
α-*TUB* (Alpha-tubulin 3-chain)	1.181 (7)	1.122 (7)	0.92 (1)	0.59*	5.00	**5**
β-*TUB* (Beta-tubulin 1-chain)	1.132 (6)	0.766 (4)	1.21 (7)	0.84*	5.67	**6**
60S (60S ribosomal protein L18)	1.245 (8)	1.287 (8)	1.07 (3)	0.63*	6.33	**7**
ACT (Actin)	1.439 (9)	2.056 (10)	1.63 (9)	0.69*	9.33	**8**
TTC7B (Tetratricopeptide repeat protein 7B)	1.602 (10)	1.972 (9)	1.98 (11)	0.78*	10.00	**9**
PsaB (Photosystem I P700 chlorophyll a apoprotein A2)	1.739 (11)	2.061 (11)	1.77 (10)	0.49	10.67	**10**

SV – Stability value; SD – Standard deviation of Cq value; r – Pearson coefficient of correlation; *$p \leq 0.01$. p-value associated with the Pearson coefficient of correlation; Ranking order is indicated in parenthesis.

4.2.3. Comprehensive ranking and determination of optimal number of RG for normalization

A comprehensive ranking, considering the 3 algorithms, was established by calculating the arithmetic mean of the ranking value of each gene given from each tool (Wang et al., 2012). The results revealed that, in grapevine leaves without stress, the three most stable genes for normalization were *UBQ*, *SAND*, *EF1*α and *AP2M* (Table IV. 5, final raking column). Noteworthy *EF1*α and *AP2M* had the same value on the ranking mean, being these two genes in the third position of the most stable reference (Table IV. 5, final raking column).

Considering the number of RGs that should be used in qPCR studies, Vandesompele J. et al. (2002) suggested to use multiple reference genes to get more accurate results (Vandesompele et al., 2002a). However, the numbers of reference genes used, and even the specific combination of them, depend on the study and type of samples analysed. In our study we decided to use the three most stable reference genes for data normalization.

4.3. Expression profile of Biomarkers candidate genes

Nowadays there is a constant need for new pathogen control strategies for grapevine crops, especially against downy mildew disease. The establishment of resistance-associated biomarker genes can be a possible solution, especially when applied to breeding programs in order to select resistant plantlets. In this study, we have selected nine metabolites putatively associated to grapevine resistance based on literature data and our preliminary metabolic results (Table IV. 3). Several studies have been performed on grapevine resistance response to *Plasmopara viticola*, and some metabolites have been pointed out as possible candidate biomarkers. Caffeic acid, fumaric acid, glutamic acid, myo-inositol, quercetin and quercetin 3-*O*-glucoside metabolites were chosen for our study due to their interesting pattern of appearance in *Vitis vinifera* cultivars, susceptible (Trincadeira) and resistant (Regent) to *Plasmopora viticola*, at different time points after inoculation (Ali et al., 2012). Moreover, these results showed that cultivar Regent had higher levels of defence-related metabolites in the first ours of infection. In relation to hexadecanoic acid, this compound might play a key role in the protection of grapevine leaves against downy mildew (Batovska et al., 2009), so it was also included in our analysis. Finally, the two remaining metabolites (catechin and epicatechin) were chosen due to their exclusive presence in susceptible *Vitis vinifera* cultivares after a metabolite comparison of all *Vitis* samples used in this work (Table IV. 1). After

metabolite selection, for each one of these metabolites, a search for their metabolic pathways was performed using KEGG, to understand in which cellular pathways they are involved and identify the enzymes responsible for their synthesis and/or degradation. After the analysis of the selected metabolic pathways and using data from literature, eleven enzymes were selected: caffeic acid *O*-methyltransferase (COMT); *p*-coumarate 3-hydroxylase (C3H); leucoanthocyanidin reductase 2 (LAR2); anthocyanidin reductase (ANR); fumarate hydratase I (FUM1); ferredoxin-dependent glutamate synthase (Fd-GOGAT); NADH-dependent glutamate synthase (NADH-GOGAT); fatty acyl-ACP thioesterase B (FatB); myo-inositol monophosphatase (IMPL1); flavonoid 3',5'-hydroxylase (F3'5'H) and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT). For every enzyme chosen, we searched for the grapevine proteins using the *Arabidopsis thaliana* homologue protein sequences. Expression analysis for every selected gene was measured by qPCR using gene-specific primer pairs (Table IV. 3). Melting curves of the selected genes were analysed to detect the absence/presence of primer dimer formation or non-specific PCR products. PCR efficiency of each primer pair was calculated through the standard curve method using the pool of all cDNA samples in a ten-fold serial dilution. To determine the expression of candidate biomarker genes, normalization was done using the three best RG given by the comprehensive ranking. Thus the expression of each one of the selected genes was normalized using a normalization factor (NF), calculated based on the expression of *UBQ*, *SAND* and *EF1*α, and since our *Vitis vinifera* cultivar of reference is Pinot noir, this cultivar was used as our control in order to understand the expression pattern of all genes in the other *Vitis* (Fig. IV. 1). Noteworthy, values below 0, around 1 an higher than 1 represent respectively, down regulation, no regulation and up-regulation of gene expression.

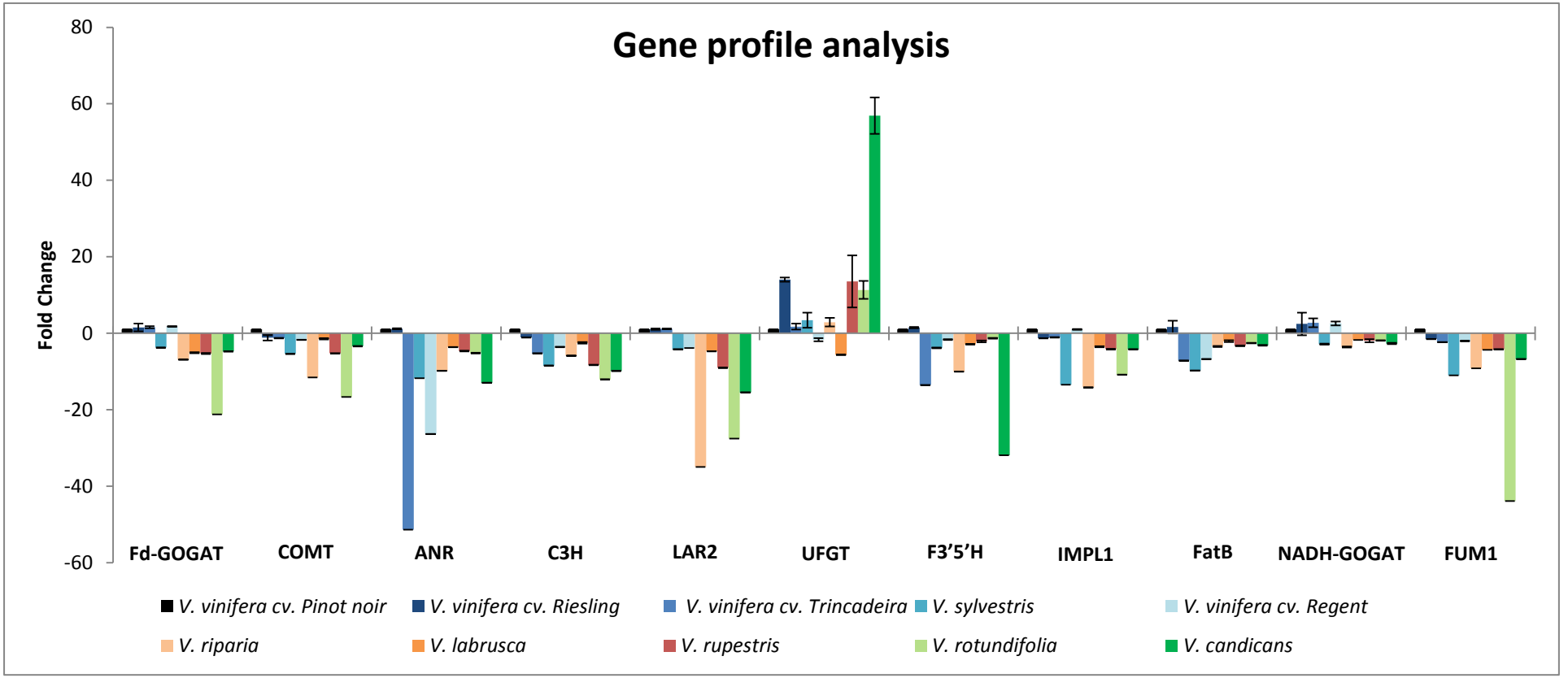

Fig. IV. 1 - Gene profile analysis of Fd-GOGAT, COMT, ANR, C3H, LAR2, UFGT, F3'5'H, IMPL1, FatB, NADH-GOGAT and FUM1 in different *Vitis* species and cultivars. Normalization was performed using a NF of the three best RGs. Fold change was calculated by comparison of all *Vitis* samples using *Vitis vinifera* cultivar Pinot noir as a control. Values below 0 represent down regulation, around 1 means basal expression and above 1 is up-regulation of gene expression.

When comparing the expression of all selected genes in the different *Vitis* species and *V. vinifera* cultivars with the cultivar Pinot noir, the only gene that is over-expressed in the majority of *Vitis* is the gene coding for the UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT), the enzyme responsible for quercetin-3-O-glucoside biosynthesis (Fig. IV. 1). This is not surprising since UFGT gene is crucial for anthocyanin biosynthesis. Since anthocyanins roles in grapevine leaves are associated to protection from environmental changes and against pathogens (Hatier and Gould, 2008; Lee and Gould, 2002), further investigations with this metabolite or coding gene should be done. Caffeic acid O-methyltransferase (COMT), *p*-coumarate 3-hydroxylase (C3H), flavonoid 3',5'-hydroxylase (F3'5'H), myo-inositol monophosphatase (IMPL1), fatty acyl-ACP thioesterase B (FatB) and fumarate hydratase I (FUM1) coding genes were all under-expressed in susceptible and resistant *Vitis*. Concerning ferredoxin-dependent glutamate synthase (Fd-GOGAT) and NADH-dependent glutamate synthase (NADH-GOGAT), the expression pattern of the coding genes in all different *Vitis* samples was equal. This is, in *Vitis vinifera* cultivars Riesling, Trincadeira and Regent there is a slightly expression for Fd-GOGAT (1.53 $\pm$ 1.02, 1.58 $\pm$ 0.28, 1.80 $\pm$ 0.08, respectively) and for NADH-GOGAT (2.44 $\pm$ 2.97, 2.72 $\pm$ 1.15, 2.57 $\pm$ 0.51, respectively). In *Vitis sylvestris*, *V. riparia*, *V. labrusca*, *V. rupestris*, *V. rotundifolia* and *V. candicans* the genes are under-expressed for Fd-GOGAT (-3.76 $\pm$ 0.04, -6,91 $\pm$ 0.03, -5.10 $\pm$ 0.11, -5.30 $\pm$ 0.12, -21.21 $\pm$ 0.02, -4.74 $\pm$ 0.07, respectively) and NADH-GOGAT (-2.87 $\pm$ 0.13, -3.57 $\pm$ 0.13, -1.76 $\pm$ 0.03, -1.96 $\pm$ 0.43, -1.90 $\pm$ 0.05, -2.68 $\pm$ 0.20, respectively). These results lead us to speculate that Fd-GOGAT and NADH-GOGAT coding genes might be constitutively over or under expressed depending of the type of accession, respectively cultivated grapevines and complex hybrids or wild species. So far, the fact that the majority of genes are overall under-expressed in susceptible and resistant grapevines leaves without any stress, make us hypothesise that these genes are only expressed in grapevine leaves during pathogen infection and maybe play a key role in plant defences after pathogen attack. A possible way to verify this is infecting the same *Vitis* species and *V. vinifera* cultivars, used in this study, and compare the obtained results.

Finally, catechin and epicatechin were the only metabolic compounds chosen from a metabolic comparison between all *Vitis* samples. Expression analysis of each biosynthesis enzymes, leucoanthocyanidin reductase 2 (LAR2) and anthocyanidin reductase (ANR), respectively, showed that as well as the other genes referred earlier, ANR is a gene with a very low expression in all *Vitis*. Interestingly, LAR2 coding gene showed under expression in all wild *Vitis* resistant to downy mildew and no expression in susceptible *Vitis* when comparing to Pinot noir. This led us to believe that constitutively this is a low copy gene and since it showed

no expression in susceptible *Vitis*, LAR2 coding gene, or even catechin, might possibly be a susceptible biomarker to downy mildew. However, further studies are necessary in order to confirm our hypothesis.

5. Conclusions

Grapevines have different degrees of resistance or susceptibility to *Plasmopora viticola*. In this study, the metabolic profiles of ten *Vitis vinifera* species and cultivars, showing different degrees of resistance towards *P. viticola*, were compared and nine distinct masses present only in resistant or susceptible to *P. viticola* were identified. Gene profile expression of genes coding for biosynthesis and degradation enzymes of key metabolic pathways were analysed by qPCR. Gene expression normalization was achieved using the three most stable reference genes (*UBQ*, *SAND* and *EF1*α or *AP2M*), of a set of eleven possible tested. Results point towards catechin as a possible biomarker of grapevine susceptibility to downy mildew.

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Concluding remarks

The European Union is the world's leader in wine production, with almost half of the total vine-growing area. Unfortunately, downy mildew disease caused by the biotrophic oomycete *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni, is currently one of the most destructive vineyard diseases affecting all *Vitis vinifera* cultivars, frequently used for wine production. Thus, if this disease is not controlled, it could have serious negative effects in several countries' economy. Some strategies have been implemented to cope with the threat caused by downy mildew. Although the majority include the intensive use of fungicides or phytochemicals, this is not the most efficient, environmental friendly and sustainable approach. A better strategy is the development of breeding programs which allow the combination of resistant traits from wild *Vitis* species into *Vitis vinifera*, while preserving their unique properties and good berry quality for wine production. However, this last approach takes years to accomplish, and to be advantageous it requires the understanding of the innate resistance mechanisms against downy mildew and the identification of resistance/susceptibility-related biomarkers. To identify specific compounds responsible for resistant or susceptible traits in different *Vitis* species and *V. vinifera* cultivars, it is crucial to make a thorough metabolic characterization in different (grape)vines. Moreover, the introgression of specific genes coding for enzymes involved in key metabolic pathways in traditional crops by breeding programs might be one of the most promising methods.

In the present study, a metabolite extraction protocol suitable for direct infusion Fourier-transform ion cyclotron-resonance mass spectrometry (DI-FTICR-MS) analysis for untargeted metabolomics was developed for *Vitis vinifera* cultivar Pinot noir leaves (Chapter II). With this extraction protocol, four fractions were obtained (methanol, water, organic and acetonitrile) and analysis by DI-FTICR-MS, using electrospray ionization (ESI) in both ionization modes, allowed the identification of 719 unique masses. This methodology increased the metabolome coverage by extracting polar and non-polar compounds, covering all major classes found in plants. Indeed, the metabolite characterization of *Vitis vinifera* cultivar Pinot noir leaves allowed the annotation of eight distinct metabolic classes (lipids, carbohydrates and carbohydrates conjugates, nucleic acids, phytochemical compounds, heterocyclic compounds, organic acids and derivatives, benzenoids and others) (Chapter III). The most represented classes were lipids, with 67% of all metabolome coverage, followed by phytochemical compounds (13%), organic acids (7%) and carbohydrates (3%). This metabolic annotation allowed a better understanding of grapevine metabolic composition, not only

important in plant defence mechanisms, but also associated with a wide range of health benefits. This approach also showed to be suitable in quality control programs of grapevine leaves commercialization, since thirteen of the most common pesticides were detected.

In the last part of this thesis (Chapter IV), a first analysis towards the identification of biomarkers (resistant or susceptible) to *Plasmopora viticola* was performed. For that, the methanol fraction of ten *Vitis* species and cultivars was analysed by DI-FTICR-MS and compared, allowing the identification of nine masses distinctly present in resistant species or susceptible cultivars. Additionally, the expression analysis of biosynthesis/degradation enzyme coding genes for two of these compounds, together with seven other reported in literature to be involved in plant defence mechanisms, were analysed by Real-time Polymerase Chain Reaction (qPCR). To achieve this, the stability of eleven reference genes for grapevine leaves without stress were also tested and Ubiquitin conjugating enzyme (*UBQ*), SAND family protein (*SAND*), elongation factor 1-alpha (*EF1*α) and adaptor protein-2 MU-adaptin (*AP2M*) were used in the qPCR normalization since they were the most stable genes. Among the analysed genes and selected compounds, the synthesis of catechin is a promising pathway to follow.

Future perspectives

Grapevine (*Vitis vinifera* L.) has always been an important part of the development of human culture and nowadays is one of the most important and cultivated fruit plant in the world, not only due to its food products, but also due to its major economical and medical importance. Unfortunately, vineyards are severely attacked by pathogens that destroy all the crops and as consequence represent major economic losses. Since the strategies used nowadays are not efficient the development of new approaches is fundamental.

This study represents the beginning of a new era for metabolic identification of resistant traits, development of new methodologies and possibly a new hope for *Vitis vinifera* against downy mildew.

The identification of different compounds, present only in resistant or susceptible *Vitis*, opened new doors towards biomarkers discovery. In the future it might be interesting to analyse the coding gene expression for the other compounds found in infected and not infected grapevine leaves, in order to understand if these compounds are either constitutively present or activated when pathogen attack. Moreover, quantification and targeted metabolomics of these compounds will contribute to advances in genetic engineering of metabolic pathways.